Diversity of Ampeloviruses in Mealybug and Soft Scale Vectors and in Grapevine Hosts from Leafroll-Affected Vineyards

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ABSTRACT

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The occurrence and diversity of *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) in the soft scales *Parthenolecanium corni* and *Pulvinaria innumerabilis* and in the mealybug *Pseudococcus maritimus* was determined in leafroll-affected vineyards in the Finger Lakes region of New York. Groups of 1 to 4 specimens were collected under loose grapevine bark and tested by reverse-transcription polymerase chain reaction (RT-PCR) for segments of the second diverged copy of the GLRaV-1 coat protein gene or

GLRaV-3 heat-shock protein 70-homologue gene. Virus-specific RT-PCR products were amplified from immature insect vectors and adult mealybugs. Single viral amplicons were obtained mostly from immature vectors (35%, 30 of 85) and dual viral amplicons from immature (16%, 10 of 61) and adult (100%, 14 of 14) mealybugs, including individuals. These observations suggested a simultaneous uptake of GLRaV-1 and GLRaV-3 by individual mealybugs. Furthermore, a comparative nucleotide sequence analysis of viral amplicons from soft scales, mealybugs, and grapevines from which vectors were collected showed identical or highly similar haplotypes, indicating that uptake of GLRaV-1 and GLRaV-3 likely occurred by direct feeding of vectors on their host plants.

Additional keywords: *Closteroviridae*, transmission, *Vitis vinifera*.

Leafroll is one of the most widespread viral diseases of grapevines. It causes significant yield losses (up to 30 to 50%), delays fruit ripening, reduces soluble solids, and increases titratable acidity in fruit juice (18,28). To date, 10 different phloem-limited filamentous viruses, identified as *Grapevine leafroll-associated viruses* (GLRaVs), have been isolated and characterized from leafroll-infected grapevines (1,27,28). These viruses belong to the genera *Closterovirus* (GLRaV-2) and *Ampelovirus* (GLRaV-1, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-9, and GLRaV-10) or have not been assigned to a genus yet (GLRaV-7) in the family *Closteroviridae* (1,27,28).

All GLRaVs are readily transmitted by propagation and grafting, and some of them (GLRaV-1, GLRaV-3, GLRaV-5, and GLRaV-9) are also vectored by several species of mealybugs (Hemiptera: Pseudococcidae) and soft scale insects (Hemiptera: Coccidae) (28). The involvement of mealybugs in the transmission of leafroll disease was suspected in the early 1960s (32) but natural leafroll spread in commercial vineyards was only reported in the early 1970s (10). Subsequently, experimental evidence of the GLRaV-3 spread by mealybugs was obtained in the laboratory (33), greenhouse (36), and vineyard (12). Overall, transmission studies showed that the mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris*, and the soft scales *Pulvinaria innumerabilis*, *P. vitis*, and *Parthenolecanium corni* are vectors of GLRaV-1 (14,19,24,38) whereas the mealybugs *H. bohemicus*, *Phenacoccus aceris*, *Planococcus citri*, *P. ficus*, *Pseudococcus calceolariae*, *P. comstocki*, *P. longispinus*, *P. maritimus*, *P. viburni*, and the soft scales *Pulvinaria vitis* and *P. innumerabilis* are vectors of GLRaV-3 (3,6,9,14,19,25,29–31,33,35,36,39).

A recent survey of vineyards in the Finger Lakes region of New York for the occurrence of leafroll-associated viruses revealed a high incidence of GLRaV-1 and GLRaV-3 (15). A concurrent survey of vineyards in the Finger Lakes region for the presence of insect vectors of GLRaV-1 and GLRaV-3 identified two soft scale species (i.e., *P. corni* and *P. innumerabilis*) and one mealybug species (i.e., *Pseudococcus maritimus*) (G. M. Loeb, *unpublished*). These coccidae and pseudococcidae are not considered major pests in local vineyards because their population density is very low and they do not cause any obvious economic damage (G. M. Loeb, *unpublished*). However, although the vector competency of *P. corni*, *P. innumerabilis*, and *P. maritimus* is well established in France, Italy, and the United States, including California and Washington (14,19,24,29,35), little is known about their contribution to the spread of GLRaV-1 and GLRaV-3 in Finger Lakes vineyards in New York.

Studying virus–vector relationships is an enticing research area. Our knowledge is rather scarce on associations among leafroll viruses and their vectors (11,28,31,38). In particular, no information is available on the viruliferous potential of soft scale and mealybug vectors in leafroll-affected vineyards. Knowledge of the potential viruliferous status of vectors is important to better understand their contribution to the efficacy of leafroll spread. The main objective of our study was to examine the occurrence and diversity of GLRaV-1 and GLRaV-3 in *P. corni*, *P. innumerabilis*, and *P. maritimus* in leafroll-diseased vineyards in New York. We report on the amplification of a segment of the second diverged copy of the GLRaV-1 coat protein (CPd2) gene and a portion of the GLRaV-3 heat-shock protein 70 homologue (HSP70h) gene in the majority of potential vectors tested, including DNA amplicons of both viruses from individual immature and adult mealybugs. We also determined and compared the nucleotide sequence of viral amplicons from vectors and their grapevine hosts, and documented limited variation. These findings provide

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new insights into the epidemiology of two major viruses associated with leafroll disease.

MATERIALS AND METHODS

Test vineyards and incidence of GLRaV-1 and GLRaV-3.

Vineyards of *Vitis vinifera* cvs. Cabernet franc, Chardonnay, Gewurztraminer, Lemberger, Pinot noir, Rkatsiteli, and Riesling and of the interspecific hybrid cv. GR7 (Buffalo × Baco noir) were selected for this study in the Finger Lakes region of New York. To characterize the incidence of GLRaV-1 or GLRaV-3 in the selected vineyards, leaf samples from the lower grapevine canopy were collected in September 2007 and 2008 by using a four-by-five-quadrat sampling strategy with a stratified regular quadrat distribution. Leaf samples were collected from each quadrat, 4 quadrats per row, every five rows, for a maximum of 20 quadrats per vineyard block. Composite samples of 3 leaves per vine and five vines per quadrat, making a total of 15 leaves per sample, were collected and tested for the presence of GLRaV-1 and GLRaV-3 by double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA). In both years, the leaf-sampling strategy was independent of leafroll disease symptoms. Leaf samples were also collected from May to July 2008 from individual grapevines from which mealybugs and soft scales were sampled and tested for their infectious status. Individual grapevines were identified by their spatial location within vineyards, with coordinates x (the row number) and y (the within-row location). For example, grapevine 6-13 was located in position 13 within row 6. We considered a virus isolate as a viral culture recovered from a single infected grapevine.

Mealybug and soft scale collection. Selected vineyards were surveyed for mealybug and soft scales in June 2007 and from May to July 2008. Immature individuals of the soft scales *P. corni* and *P. innumerabilis* and of the mealybug *P. maritimus* were collected in June 2007 and in May to June 2008. Adults of *P. maritimus* were sampled in July 2008. Soft scales and mealybugs were collected randomly under cracked bark of 1- and sometimes 2-year-old wood. Collected specimens were placed in 1.5-ml microfuge tubes and kept in RNAlater storage solution (Ambion, Inc., Austin, TX) at -20°C until further processing for total RNA extraction. Soft scales (S) and mealybugs (M) were identified by the spatial location of their grapevine host within vineyards. For example, soft scale S-6-13-B was collected on grapevine in position 13 within row 6 of vineyard B and mealybug M-1-1-7-G was found on grapevine in position 17 within row 1 of vineyard G.

Virus detection in grapevine leaf tissue by DAS-ELISA. GLRaV-1 and GLRaV-3 were detected by DAS-ELISA in grapevine leaf tissue with specific antibodies (Bioreba, Reinach, Switzerland). A portion of 15 stacked leaves was torn and ground in 200 mM Tris-HCl, pH 8.2; 140 mM NaCl; 2% polyvinylpyrrolidone 40; and 0.05% Tween 20 at a 1:10 ratio (wt/vol) using a semiautomated ball-bearing HOMEX tissue homogenizer (Bioreba). Test conditions were in accordance with the manufacturer's instructions (Bioreba). Substrate hydrolysis was recorded at 405 nm with an absorbance BioTek ELx808TM microplate reader (BioTek, Winooski, VT). Samples were considered positive if their optical density at 405 nm readings were at least twice those of healthy controls.

Detection of partial genomic segments of GLRaV-1 and GLRaV-3 in insects and grapevines by reverse-transcription polymerase chain reaction. Mealybugs, soft scales, and grapevine leaf samples were assayed for GLRaV-1 and GLRaV-3 by reverse-transcription polymerase chain reaction (RT-PCR) with total RNA and appropriate primers. For RNA extraction, individual or groups of 2 to 4 insects, or leaf tissue (100 mg), were placed in a 2.0-ml microfuge tube, dipped in liquid nitrogen, and disrupted with a TissueLyser homogenizer (Qiagen, Valencia, CA) for 2 min at 30 Mhz in the presence of one stainless steel

bead (5 mm in diameter). Total RNA was extracted from homogenized material using the RNeasy mini plant kit (Qiagen).

A segment of the CPd2 gene of GLRaV-1 and a portion of the HSP70h gene of GLRaV-3 were characterized by RT-PCR from total RNA with primers described previously (29). A primer pair specific to the genus *Vitis* 18S ribosomal gene (16) was used as internal control to amplify the corresponding mitochondrial RNA from grapevine leaf tissue in standard and multiplex RT-PCR. Similarly, primer pairs for the nuclear small-subunit ribosomal RNA gene (SSU rRNA) (8) and rDNA internal transcribed spacer (ITS) region (4) were used in standard and multiplex RT-PCR assays as internal controls for mealybug and soft scale insects, respectively.

RT-PCR was carried out using the OneStep kit (Qiagen) with Ominiscript and Sensiscript RTases, HotStart Taq DNA polymerase, and 50 pmol of specific primers in a 50- μl final volume according to the manufacturer's protocol. Single-tube RT-PCR used a 30-min heating step at 50°C and a 15-min heating step at 95°C followed by 35 cycles of 30 s of melting at 94°C , 1 min of annealing at 50°C , and 1 min of elongation at 72°C , with a final extension of 10 min at 72°C . The reaction products were resolved by electrophoresis in 1.5% agarose gels in 40 mM Tris-acetate and 10 mM EDTA, pH 8.0; stained with ethidium bromide; and subsequently visualized under UV light.

Alternatively, GLRaV-1 and GLRaV-3 were characterized in grapevine leaf tissue by immunocapture (IC)-RT-PCR. Virus particles were immunocaptured by loading plant extracts in microfuge tubes precoated with antibodies specific to GLRaV-1 and GLRaV-3 (Bioreba). After three washes with phosphate buffer saline supplemented with 5% Tween-20, virus particles were suspended in 10 μl of sterile water and denatured by heating for 5 min at 70°C and chilling on ice. One microliter was used in one-step RT-PCR assays to characterize the GLRaV-1 CPd2 gene and the GLRaV-3 HSP70h gene as described above.

Viral sequence determination and analysis. DNA amplicons obtained by RT-PCR or IC-RT-PCR from grapevine leaf tissue, soft scales, and mealybugs were extracted from agarose gels with the QIAquick purification kit (Qiagen) and sequenced bidirectionally using the Big Dye Terminator kit, AmpliTaq-FS DNA polymerase, and an Applied Biosystem Automated 3730xl DNA Analyzer at the DNA Sequencing facility at Cornell University in Ithaca, NY. Sequences were analyzed and compared using the DNASTAR Lasergene v7.2 software package. The program CLUSTAL W was used for alignment of nucleotide sequences (37). Phylogenetic relationships were determined with the neighbor-joining (NJ) (34) and maximum parsimony (MP) methods using the PHYLIP version 3.6 program (13). The robustness of the inferred evolutionary relationships was assessed by 1,000 bootstrap replicates.

RESULTS

One vineyard of an interspecific hybrid cultivar and 12 vineyards of *V. vinifera* cultivars were selected for this study (Table 1). During leaf sampling in September, symptoms of leafroll disease were visible in most of these vineyards, with leaf cupping and reddening in red-fruited *V. vinifera* cultivars and cupping and a slight chlorosis in white-fruited *V. vinifera* cultivars. The occurrence of GLRaV-1 and GLRaV-3 was confirmed by DAS-ELISA in vineyards with symptomatic vines (Table 1). Absorbance values were clearly higher for infected (0.79 ± 0.16 for GLRaV-1 and 0.68 ± 0.21 for GLRaV-3) compared with healthy (0.01 ± 0.006) samples after substrate hydrolysis for 1 h. Virus incidence varied from moderate (20 to 35%) to high (40 to 44%) or very high (70 to 85%) in three (C, H, and K), two (F and L), and four (B, D, G, and I) of the vineyards surveyed, respectively (Table 1). The two target viruses (i.e., GLRaV-1 and GLRaV-3) were not found in vineyards E and M,

and vineyards A and J were not surveyed by DAS-ELISA but coccidae and pseudococcidae were collected for vector studies (Table 1). Single infection by GLRaV-1 occurred in vineyards C, H, I, and K, and single infection by GLRaV-3 in vineyard D, as shown by DAS-ELISA. Dual infection by GLRaV-1 and GLRaV-3 was detected in vineyards B, F, G, and L, with mixed infection occurring in vineyards G and L (Table 1). These data were confirmed in individual grapevines by multiplex RT-PCR (Fig. 1). A 401-bp segment of the GLRaV-1 CPd2 gene was amplified from a single grapevine in vineyard C (Fig. 1, lane 8) and a 546-bp segment of the GLRaV-3 HSP70h gene was obtained from a single grapevine in vineyard D (Fig. 1, lane 9). Co-amplification of a segment of the GLRaV-1 CPd2 gene and GLRaV-3 HSP70h gene in a single grapevine in vineyard L confirmed the occurrence of mixed infection (Fig. 1, lane 10). As internal control, an 844-nucleotide (nt)-long RT-PCR product for the *Vitis* 18S RNA was amplified in all the infected grapevine samples tested (Fig. 1, lanes 8 to 10), as well as in total RNA from leaves of a healthy grapevine from vineyard N (Fig. 1, lane 7).

Mealybugs and soft scales were collected over two consecutive years in the 13 commercial vineyards selected for this study, including the 9 vineyards affected by GLRaV-1 or GLRaV-3. Immature vectors were collected in May to June and adults were sampled in late July. Collected specimens were assayed for the occurrence of the two target ampeloviruses with overall more mealybugs (76%, 75 of 99) than soft scales (24%, 24 of 99) (Table 2). The difference in population between coccidae and pseudococcidae is due to the fact that more mealybugs than soft scales were found in 2008; it did not reflect a general trend in Finger Lakes vineyards because more soft scales than mealybugs were observed in previous years (G. M. Loeb, *unpublished*). The reason for a more abundant mealybug population in 2008 is unclear. For our survey, more immature individuals (86%, 85 of 99) than adults (14%, 14 of 99) were collected and tested (Table 2) because the former are more efficient vectors of ampeloviruses than the latter development stage (31,38). Individuals represented 83% (20 of 24) of the immature soft scales, 23% (14 of 61) of the immature mealybugs, and 100% (14 of 14) of the adult mealybugs tested (Table 2).

A 401-bp segment of the GLRaV-1 CPd2 gene was obtained from a single soft scale (Fig. 1, lane 1) and a single mealybug (Fig. 1, lane 4) in vineyards K and F, respectively. A 546-bp segment of the GLRaV-3 HSP70h gene was amplified from a single soft scale (Fig. 1, lane 2) and a single mealybug (Fig. 1, lane 5) in vineyards A and F, respectively. No viral DNA amplicon

was obtained from a soft scale (Fig. 1, lane 3) and a mealybug (data not shown) collected on healthy grapevines in vineyards E and M, respectively. Amplicons of the GLRaV-1 CPd2 gene and the GLRaV-3 HSP70h gene were both obtained from a single adult mealybug from vineyard B (Fig. 1, lane 6), suggesting a simultaneous uptake of both ampeloviruses by individual mealybugs. The two rDNA markers were used successfully to normalize RT-PCR assays with an $\approx 1,500$ -bp DNA fragment of the rDNA ITS amplified from mealybugs and an ≈ 700 -bp DNA fragment of the SSU rDNA obtained from soft scales (data not shown).

Viral amplicons were detected in the majority of mealybugs tested by RT-PCR (63%, 47 of 75); nearly 1/3 had amplicons of both ampeloviruses (32%, 24 of 75), 1/5 had amplicons of GLRaV-3 (21%, 16 of 75), and nearly 1/10 had amplicons of GLRaV-1 (9%, 7 of 75) (Table 3). Mealybugs with an amplicon specific to the GLRaV-1 CPd2 gene segment were found in vineyard F and those with an amplicon specific to the GLRaV-3 HSP70h gene segment were found in vineyards B, F, and G (Table 3). Mealybugs with amplicons of the two target viruses were found in vineyard B, F, and G (Table 3). All individual adult mealybugs tested (100%, 14 of 14) had amplicons of both ampeloviruses and individual immature mealybugs had mainly GLRaV-3 amplicons (43%, 6 of 14) followed by amplicons of both ampeloviruses (21%, 3 of 14) and GLRaV-1 amplicons (7%, 1 of 14) (Table 3).

Fig. 1. Agarose gel analysis of DNA amplicons obtained by multiplex reverse-transcription polymerase chain reaction from total RNA of insects or grapevine leaves infected by *Grapevine leafroll-associated virus 1* (GLRaV-1) (401 bp) and GLRaV-3 (546 bp), and primer pairs specific to each virus. A primer set specific to the *Vitis* sp. 18S ribosomal RNA was also used with total grapevine RNA to amplify an 844-bp fragment. Lane 1: Immature soft scale from vineyard K; lane 2: immature soft scale from vineyard A; lane 3: immature soft scale from a healthy grapevine from vineyard E; lane 4: immature mealybug from vineyard F; lane 5: immature mealybug from vineyard F; lane 6: immature mealybug from vineyard L; lane 7: healthy grapevine; lane 8: grapevine from vineyard C; lane 9: grapevine from vineyard D; and lane 10: grapevine from vineyard G. Lanes M: 100-bp DNA size standard (Promega Corp., Madison, WI). DNA products were resolved by electrophoresis on 1% agarose gels.

TABLE 1. Occurrence of *Grapevine leafroll-associated virus 1* (GLRaV-1) or GLRaV-3 in commercial vineyards in the Finger Lakes region of New York

Vineyard	Genotype	N ^a	No of vineyard quadrat positive for ^b				Incidence (%) ^c
			No virus	GLRaV-1	GLRaV-3	GLRaV-1+GLRaV-3	
A	GR7	nd	nd	nd	nd	nd	na
B	Lemberger	16	3	10	3	0	81
C	Lemberger	10	8	2	0	0	20
D	Cabernet franc	10	2	0	8	0	80
E	Cabernet franc	20	20	0	0	0	0
F	Chardonnay	16	11	5	2	0	44
G	Chardonnay	20	3	2	6	9	85
H	Chardonnay	12	8	4	0	0	33
I	Gewurztraminer	20	6	14	0	0	70
J	Rkatsiteli	nd	nd	nd	nd	nd	na
K	Riesling	20	13	7	0	0	35
L	Pinot noir	20	12	6	0	2	40
M	Pinot Noir	20	0	0	0	0	0

^a Number of quadrats of five grapevines tested per vineyard.

^b Data represent the number of quadrats for which grapevine leaf samples tested negative (no virus) or positive for GLRaV-1 or GLRaV-3 in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA); nd = not determined and na = not applicable.

^c Incidence of viruses calculated as the number of quadrats reacting positively to GLRaV-1 and/or GLRaV-3 in DAS-ELISA over the total number of quadrats tested.

Most of the soft scales tested had no viral amplicon (71%, 17 of 24) (Table 4); only 29% (7 of 24) of them had amplicons specific to the GLRaV-1 CPd2 gene segment (21%, 5 of 24) or to the GLRaV-3 HSP70h gene segment (8%, 2 of 24) (Table 4). Co-amplification of GLRaV-1 and GLRaV-3 DNA products was not obtained in any of the soft scales tested. Soft scales with GLRaV-1 amplicons were found in vineyards C and F and those with GLRaV-3 amplicons were found in vineyards A and F (Table 4). In the case of individual immature soft scales, viral amplicons were obtained in one-fourth of the specimens tested (25%, 5 of 20), mainly GLRaV-1 amplicons (15%, 3 of 20) but also GLRaV-3 amplicons (10%, 2 of 20) (Table 4).

Across all mealybugs and soft scales tested, data compiled from Tables 3 and 4 indicated that viral DNA fragments were amplified from the majority of the specimens (55%, 54 of 99). Viral amplicons were obtained in nearly half of the immature vectors (47%, 40 of 85) and in all of the adults (100%, 14 of 14) tested. Immature individuals had mostly GLRaV-3 amplicons (21%, 18 of 85) followed by GLRaV-1 amplicons (14%, 12 of 85) and amplicons of both ampeloviruses (12%, 10 of 85), whereas

all adults had dual viral amplicons (Tables 3 and 4). A χ^2 analysis indicated that the frequency distribution of viral amplicons was dependent on the development stage of mealybugs (adult versus immature individuals) ($P < 0.001$). Also, 61% (54 of 88) of the vectors tested from leafroll-affected vineyards had viral amplicons and those collected in healthy vineyards (E and M) had no viral amplicons (Tables 3 and 4). One soft scale with a GLRaV-3 amplicon was also detected in vineyard A established with interspecific hybrid cv. GR7, for which leafroll disease infection is latent.

The majority of individual vectors tested had viral amplicons (58%, 28 of 48) and the frequency distribution was 17% (8 of 48) with GLRaV-3 amplicons, 8% (4 of 48) for GLRaV-1 amplicons, and 33% (16 of 48) with amplicons of both GLRaV-1 and GLRaV-3 (Tables 3 and 4). Single immature soft scales and mealybugs with GLRaV-3 amplicons were detected in vineyards A and F (Table 3) and in vineyards B, F, and G, respectively (Table 4). Single immature and adult mealybugs with GLRaV-1 and GLRaV-3 amplicons were identified in vineyards B, F, and G (Table 4).

TABLE 2. Mealybugs and soft scales collected in Finger Lakes vineyards and tested for the occurrence of *Grapevine leafroll-associated virus 1* (GLRaV-1) and GLRaV-3 by reverse-transcription polymerase chain reaction (RT-PCR)

Vineyard	Genotype	Virus status ^a	Insect ^b	Immature ^c	Adult
A	GR7	nd	Soft scales	4 (4)	0
B	Lemberger	GLRaV-1/GLRaV-3	Mealybugs	17 (4)	13 (13)
			Soft scales	1 (1)	0
C	Lemberger	GLRaV-1	Soft scales	4 (2)	0
D	Cabernet franc	GLRaV-3	Mealybugs	9 (0)	0
			Soft scales	1 (1)	0
E	Cabernet franc	None	Mealybug	1 (1)	0
			Soft scales	1 (1)	0
F	Chardonnay	GLRaV-1/GLRaV-3	Mealybugs	9 (3)	1 (1)
			Soft scales	2 (2)	0
G	Chardonnay	GLRaV-1/GLRaV-3	Mealybugs	23 (4)	0
H	Chardonnay	GLRaV-1	Soft scales	4 (4)	0
I	Gewurztraminer	GLRaV-1	Soft scales	1 (1)	0
J	Rkatsiteli	nd	Soft scales	5 (3)	0
K	Riesling	GLRaV-1	Soft scales	1 (1)	0
L	Pinot noir	GLRaV-1/GLRaV-3	Mealybugs	1 (1)	0
M	Pinot Noir	None	Mealybugs	1 (1)	0
Total	...	...	...	85 (34)	14 (14)

^a Occurrence of viruses in grapevines was determined by double-antibody sandwich enzyme-linked immunosorbent assay and RT-PCR using specific antibodies and primers, respectively; nd = not determined.

^b Insect species were identified based on morphological characteristics. The number of *Parthenolecanium corni* and *Pulvinaria innumerabilis* was combined because the two soft scale species were distinguished in 2007 but not in 2008.

^c Number of immature and adult insects tested by RT-PCR is indicated. Immature individuals were collected on 14, 19–21, and 26 June 2007 and on 20 May, 5 June, and 12 June 2008. Adults were collected on 29 July 2008. The number of single immature and adult insects tested is indicated in parenthesis.

TABLE 3. Occurrence of *Grapevine leafroll-associated virus 1* (GLRaV-1) or GLRaV-3 in immature and adult mealybugs collected on grapevines in Finger Lakes vineyards

Vineyard	Virus status ^a	Ds ^b	N ^c	Mealybugs positive for ^d			
				No virus	GLRaV-1	GLRaV-3	GLRaV-1+GLRaV-3
B	GLRaV-1/GLRaV-3	I	17 (4)	12 (1)	0	5 (3)	0
		A	13 (13)	0	0	0	13 (13)
D	GLRaV-3	I	9 (0)	9 (0)	0	0	0
E	None	I	1 (1)	1 (1)	0	0	0
F	GLRaV-1/GLRaV-3	I	9 (3)	0	7 (1)	1 (1)	1 (1)
		A	1 (1)	0	0	0	1 (1)
G	GLRaV-1/GLRaV-3	I	23 (4)	4	0	10 (2)	9 (2)
L	GLRaV-1/GLRaV-3	I	1 (1)	1 (1)	0	0	0
M	None	I	1 (1)	1 (1)	0	0	0
Total	...	...	75 (28)	28/75 (4)	7/75 (1)	16/75 (6)	24/75 (17)
Incidence (%)	...	...	...	37	9	21	32

^a Occurrence of viruses in grapevines was determined by double-antibody sandwich enzyme-linked immunosorbent assay and RT-PCR.

^b Development stage of insect vectors, immature (I) versus adult (A), is indicated.

^c Number of specimens tested. Single immature and adult insects are indicated in parenthesis.

^d Data represent the number of soft scales with no viral amplicons (no virus) or amplicons of GLRaV-1, GLRaV-3, and GLRaV-1 + GLRaV-3, as shown by reverse-transcription polymerase chain reaction (RT-PCR). Immature insects were collected in June 2007 and May to June 2008. Adults were collected in July 2008.

Amplicons of the GLRaV-1 CPd2 gene and GLRaV-3 HSP70h gene from individual soft scales and mealybugs and their corresponding grapevine hosts were sequenced. This comparative study focused essentially on vineyards B, F, and G, for which mixed virus infection and relatively high populations of insect vectors compared with the other vineyards surveyed were documented (Table 2). Viral haplotypes in vectors were determined by sequencing RT-PCR products from specimens collected in June and July whereas viral haplotypes in grapevines were determined by sequencing RT-PCR or IC-RT-PCR products from leaf tissue collected in July and September. Nucleic acid sequences determined in this study covered a 205-nt portion of the GLRaV-1 CPd2 gene (15 haplotypes: 2 from soft scales, 6 from mealybugs, and 7 from grapevines) and a 468-nt segment of the GLRaV-3 HSP70h gene (25 haplotypes: 2 from soft scales, 14 from mealybugs and 9 from grapevines). Nucleotide sequences that were unique were made available in GenBank as accession numbers FJ883754 to FJ883769. For GLRaV-1, a high nucleotide sequence identity was found within the CPd2 gene segment characterized from mealybugs (95.1 to 100%) collected in vineyards F and G and from infected grapevine leaf tissue (93.2 to 100%) in vineyard G. Similarly, a very high nucleotide sequence identity was obtained for viral amplicons from two soft scales (99.2%) and two infected grapevines (99.0%) in vineyard B. A pairwise nucleotide sequence analysis further showed identical GLRaV-1 haplotypes in two mealybugs (M-5-48 and M-5-63) and three grapevines (G-1-48, G-5-12, and G-5-78) in vineyard G. Similarly, identical GLRaV-1 haplotypes were found in two scales (S-16 and S17) and two grapevines (Lem 1 and Lem 2) in vineyard B. These results were consistent with the notion that uptake of GLRaV-1 by mealybugs likely occurred by direct feeding on their grapevine host or on other grapevines from their vineyard of origin. For GLRaV-3, a high nucleotide sequence identity was obtained within the HSP70h gene segment determined from vectors (93.8 to 100%) and from infected grapevine leaf tissue (94.0 to 100%) in vineyards B and G. Furthermore, regardless of the source of viral amplicons (i.e., vectors versus grapevines), a comparative nucleotide sequence analysis showed no variability among datasets from samples collected in June (93 to 100%, mealybugs and soft scales), July (94.7 to 100%, mealybugs and grapevines), and September (94.9 to 99.4%, grapevines). Also, a pairwise nucleotide sequence analysis showed identical GLRaV-3 haplotypes in seven mealybugs (M-1-18, M-1-78, M-4-6, M-5-15, M-5-63, M-5-78, and M-5-80) and five grapevines (G-1-12, G-1-15, G-1-48, G-5-63, and G-5-78) in vineyard G. In the same vineyard, identical GLRaV-3 haplotypes were found for other mealybug and grapevine combinations (M-5-48, G-1-63, and G-

5-48; and M-5-33 and G-1-78). These results indicated that, as for GLRaV-1, uptake of GLRaV-3 by mealybugs likely occurred by direct feeding on their grapevine host or on other grapevines within their vineyard of origin.

Phylogenetic relationships were inferred using the NJ and MP methods for the 40 viral haplotypes determined in this study, together with corresponding sequences of the type strains B4L of GLRaV-1 (GenBank accession number AF195822) and NY-1 of GLRaV-3 (GenBank accession number AF039204) (Fig. 2). Also, corresponding sequences of GLRaV-1 (GenBank accession numbers FJ195739 to FJ195741) and GLRaV-3 (GenBank accession numbers FJ195745 to FJ195749) isolates from grapevines in Finger Lakes vineyards that were determined previously (15) and GLRaV-3 strains GP-18 (GenBank accession number EU259806) and MT48-2 (GenBank accession number AJ748519) were added. Trees calculated with the NJ and MP methods were closely similar (data not shown). GLRaV-1 haplotypes clustered into two groups (Fig. 2A), which were previously designated B4L and Lem (15). GLRaV-3 haplotypes clustered into three groups designated NY-1, GP-18, and MT48-2 (15) with those characterized in this study clustering only in groups NY-1 and GP-18 (Fig. 2B). The same grouping of virus isolates was obtained with phylograms obtained from deduced amino acid sequences (68 amino acids of the GLRaV-1 CPd2 protein and 156 amino acids of the GLRaV-3 HSP70h protein). For GLRaV-1, the intragroup nucleotide sequence identity was 85.9 to 100% for B4L and 99 to 100% for Lem, and the intergroup identity was 80.0 to 83.9%. For GLRaV-3, the intragroup nucleotide sequence identity was 99.1 to 100% for NY-1 and 94.0 to 100% for GP18, and the intergroup identity was 90.8 to 96.4%. The intra- and intergroup nucleotide sequence identities were not determined for MT48-2 because none of the haplotypes characterized in this study clustered in this group. These results inferred no grouping of genetic variants based on their origin (i.e., vectors versus grapevine hosts) because GLRaV-1 (Fig. 2A) and GLRaV-3 (Fig. 2B) haplotypes from soft scales and mealybugs clustered with those from grapevines, confirming data from multiple sequence analyses. Notwithstanding, GLRaV-3 haplotypes from mealybugs collected in June on grapevine 1-18 in vineyard B belonged to group GP18 whereas those from mealybugs collected on the same grapevine host in July belonged to group NY-1 (Fig. 2B). Similarly, the GLRaV-3 haplotype from a mealybug collected in June on grapevine 1-78 in vineyard G belonged to cluster NY-1 whereas the one characterized from the same grapevine in September belonged to cluster GP-18. These observations suggested a temporal dynamic of GLRaV-3 haplotype accumulation in some grapevines and, as a result, an uptake of distinct GLRaV-3 haplotypes by mealybugs over time.

TABLE 4. Occurrence of *Grapevine leafroll-associated virus 1* (GLRaV-1) or GLRaV-3 in immature soft scales collected on grapevines in Finger Lakes vineyards

Vineyard	Virus status ^a	N ^b	Immature soft scales positive for ^c			
			No virus	GLRaV-1	GLRaV-3	GLRaV-1+GLRaV-3
A	nd	4 (4)	3 (3)	0	1 (1)	0
B	GLRaV-1/GLRaV-3	1 (1)	1 (1)	0	0	0
C	GLRaV-1	4 (2)	1 (1)	3 (1)	0	0
D	GLRaV-3	1 (1)	1 (1)	0	0	0
E	None	1 (1)	1 (1)	0	0	0
F	GLRaV-1/GLRaV-3	2 (2)	0	1 (1)	1 (1)	0
H	GLRaV-1	4 (4)	4 (4)	0	0	0
I	GLRaV-1	1 (1)	1 (1)	0	0	0
J	nd	5 (3)	5 (3)	0	0	0
K	GLRaV-1	1 (1)	0	1 (1)	0	0
Total	...	24 (20)	17/24 (15)	5/24 (3)	2/24 (2)	0/24 (0)
Incidence (%)	...	...	71	21	8	0

^a Occurrence of viruses in vineyards was determined by double-antibody sandwich enzyme-linked immunosorbent assay and RT-PCR; nd = not determined.

^b Number of specimens tested. Single immature insects are indicated in parenthesis.

^c Data represent the number of soft scales with no viral amplicon (no virus) or amplicons for GLRaV-1, GLRaV-3, and GLRaV-1 + GLRaV-3, as shown by reverse-transcription polymerase chain reaction (RT-PCR). Immature insects were collected in June 2007 and May to June 2008.

DISCUSSION

We characterized the occurrence and diversity of GLRaV-1 CP2 or GLRaV-3 HSP70h gene segments in the soft scales *P. corni* and *P. innumerabilis* and in the mealybug *P. maritimus* from Finger Lakes vineyards of New York. RT-PCR assays revealed viral amplicons in most specimens, including those assayed individually, from grapevines infected with GLRaV-1 or GLRaV-3.

To our knowledge, this is the first survey of soft scales and mealybugs for their potential viruliferous status in leafroll-diseased vineyards. In previous studies, GLRaV-3 was detected by IC-RT-PCR in individual mealybugs used in laboratory transmission assays (38) and by RT-PCR in groups of 10 mealybugs maintained on infected grapevine shoots (2). We used a highly sensitive RT-PCR assay to reliably test individuals or groups of 2 to 4 specimens collected in commercial vineyards for the pres-

Fig. 2. Phylogenetic trees reconstructed from partial nucleotide sequences of the **A**, second diverged copy of the coat protein gene of *Grapevine leafroll-associated virus 1* (GLRaV-1) and **B**, heat-shock protein 70 homologue gene of GLRaV-3 from mealybugs, soft scales, and grapevines by the neighbor-joining method with 1,000 bootstrap replicates. Numbers above critical branches are significant bootstrap values (>75%). The scale bar represents a relative genetic distance of 0.1. Sequence accessions characterized in this study are indicated with their organism of origin (S = soft scale, M = mealybug, and G = grapevine) and the location of grapevine host (1-15, 1-18, and so on) within vineyards (A, B, F, and G). GLRaV-3 haplotypes from mealybugs and grapevines that were collected at different times (June, July, or September) and belong to distinct phylogenetic groups are indicated by triangles.

ence of GLRaV-1 and GLRaV-3. A comparative study of partial GLRaV-1 CPd2 and GLRaV-3 HSP70h gene sequences from vectors and infected grapevines showed no differentiation based on the origin of the viral amplicons. These results are consistent with the notion that soft scale and mealybug vectors are unlikely to have a bottleneck effect on the genetic variability of GLRaV-1 and GLRaV-3 in terms of a selective transmission of certain haplotypes from grapevine to grapevine.

Documenting the potential viruliferous status of indigenous soft scales and mealybugs from vineyards provides valuable insights into virus uptake but does not inform us about virus acquisition and infectious potential of vectors. In order for virus transmission to occur, soft scales and mealybugs need to feed on infected grapevines to acquire GLRaV-1 or GLRaV-3. They further need to retain virus particles for an extended time (31,38) and then release virus particles following subsequent feeding on healthy grapevines. Experimental and field transmission studies documented the role of *P. corni*, *P. innumerabili*, and *P. maritimus* in the spread of GLRaV-1 or GLRaV-3 in France, Italy, and the United States, including California and Washington (14,19,24,29,35). Also, a single mealybug nymph is capable of infecting a healthy grapevine with GLRaV-3 (11). Thus, there is little doubt that some of the specimens assayed for the GLRaV-1 CPd2 gene and GLRaV-3 HSP70h gene are competent for acquisition and transmission of these two ampeloviruses in Finger Lakes vineyards of New York although their population density is extremely low.

Our findings showed a preferential association between soft scales and GLRaV-1 and, to a lower extent, between mealybugs and GLRaV-3. These observations are consistent with *P. corni* transmitting GLRaV-1 (14,24,35), *P. maritimus* transmitting GLRaV-3 (19), and *P. innumerabilis* transmitting GLRaV-1 and GLRaV-3 (3,14). However, the fact that immature and adult *P. maritimus* individuals with amplicons of both ampeloviruses were identified in this study raises the question of the vector ability of this mealybug species. To date, there is evidence for its capacity to transmit GLRaV-3 (19,29) but limited information is available for its competency at transmitting GLRaV-1 (19). Our results call for additional transmission studies to address the acquisition and transmissibility of GLRaV-1 by *P. maritimus*. They suggest also that mere uptake of ampeloviruses may not be sufficient for transmission and that virus particles may conceivably need to be retained stably at specific sites through binding to ligands for successful acquisition and transmission to occur following ingestion by vectors.

The fact that a significant difference in frequency distribution of viral amplicons was found between immature and adult mealybugs raises the question of a differential behavior of immature individuals and adults. By analogy with the seasonal phenology of mealybugs in California and Washington (17,21), virus uptake by immature individuals in Finger Lakes vineyards likely occurred by feeding on phloem tissue of infected grapevines in early spring after the eggs hatched whereas it was achieved by adults in late June or early July after nymphs have completed their development. Although first-instar immature mealybugs are more efficient vectors of GLRaV-3 than third-instar immature and adult mealybugs, a differential transmission ability of distinct *Ampelovirus* spp. by immature and adult vectors is not known (31,38). Therefore, the apparent dynamic of virus uptake observed in this study is likely associated with a differential virus multiplication in infected grapevines. Interestingly, GLRaV-1 was not detected in grapevine leaf samples collected in vineyard D in July (0%, 0 of 14) whereas GLRaV-3 was poorly detected (21%, 3 of 14). In contrast, both viruses were readily detected in leaf samples collected in September (100%, 14 of 14) from the same vineyard. Similar virus detection experiments carried out over time in an experimental vineyard in the Alsace region in France indicated that GLRaV-3 is detectable by DAS-

ELISA starting in late May whereas GLRaV-1 was often not detectable until mid-June or later, regardless of whether grapevines subjected to single or dual infection were surveyed (M. Fuchs, unpublished).

The transmission of GLRaV-1 and GLRaV-3 by mealybug and soft scale vectors contributes to the spatiotemporal increase of leafroll-diseased grapevines in commercial vineyards (5–7,12,20,22,23,26,30). Spread of GLRaV-3 usually starts slowly but progresses more rapidly as diseased grapevines cluster (6,8,20,23). No information is available on the spatiotemporal spread of GLRaV-1 although a trend similar to that of GLRaV-3 is anticipated. The extent of mealybug contribution to the spread of leafroll disease in vineyards relies mainly on first instars, which are the most mobile stage of mealybugs because immature and female mealybugs are apterous and adult males are winged but do not feed because they lack mouthparts (17,21). However, first instars do not walk very far and only rarely reach adjacent grapevines by crawling (21). The fact that fewer immature individuals than adults had viral amplicons in our study could explain the limited efficiency of these vectors at spreading ampeloviruses, confirming previous observations (3,11,19,24,31,35,38,39). Nevertheless, first instars can be dispersed by wind although a marked decline in movement efficacy is documented with increasing distance from the source grapevine (21). Our comparative sequence analyses showed identical GLRaV-1 and GLRaV-3 haplotypes in some insect vectors and in infected grapevine leaf tissue from which they were collected. These results support a limited mobility of mealybugs and soft scales within leafroll-diseased vineyards in New York. If insect vectors were landing on grapevines from which they were collected as a consequence of airborne dispersal from adjacent or distant leafroll-diseased vineyards, a higher nucleotide sequence variability than observed would have been expected within viral haplotypes, as shown in our previous genetic diversity study (15). Because the nucleotide sequence variation of the targeted GLRaV-1 and GLRaV-3 gene segments was limited and no vectors with viral amplicons were found in vineyards with no history of leafroll disease, a wind dispersal of viruliferous mealybugs and soft scales does not seem prevalent in Finger Lakes vineyards. Rather, our observations support a restricted mobility of insect vectors and, hence, limited between-vineyard dissemination of GLRaV-1 and GLRaV-3 in New York. In other viticultural regions, the situation might be different because dispersal of viruliferous mealybugs by the wind, field workers, or harvesting machinery seems to be a serious concern for the spread and management of leafroll disease (20,26).

Prior to our study, soft scales and mealybugs were not considered important pests in Finger Lakes vineyards. Because we documented a prevalence of specimens with amplifiable *Ampelovirus* gene segments in local leafroll-infected vineyards, pest management strategies may need to be deployed against the insect vectors to mitigate the impact of GLRaV-1 and GLRaV-3 in New York. In addition, a more careful selection of certified, virus-tested planting material is strongly recommended as well as a timely rouging of leafroll-infected plants to reduce the possibility of within- and between-vineyard spread of leafroll disease.

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