Vitis californica and *Vitis californica* × *Vitis vinifera* Hybrids are Hosts for Grapevine leafroll-associated virus-2 and -3 and Grapevine virus A and B

V. A. Klaassen, S. T. Sim, and G. S. Dangl, Foundation Plant Services, and F. Osman, M. Al Rwahnih, A. Rowhani, and D. A. Golino, Foundation Plant Services and Department of Plant Pathology, University of California, Davis 95616

Abstract

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Vitis and non-*Vitis* spp. surrounding nine Napa Valley vineyards were surveyed for Grapevine leafroll-associated virus (GLRaV)-1 to -5 and -9, Grapevine virus A (GVA), Grapevine virus B (GVB), and Grapevine virus D (GVD). *Vitis* spp. from three riparian areas not adjacent to vineyards were also included. DNA fingerprinting and probability analyses indicated that the *Vitis* samples consisted primarily of *Vitis californica* followed by *V. californica* × *V. vinifera* hybrids. Single and mixed infec-

tions of GLRaV-2, -3, GVA, or GVB were detected by conventional or quantitative reverse-transcription polymerase chain reaction in 6 of the 66 *V. californica* and 11 of the 19 *V. californica* × *V. vinifera* hybrids. GLRaV-1, -4, -5, -9, and GVD were not detected. Phylogenetic analysis of GLRaV-2 and -3 partial coat protein gene nucleotide sequences indicated that the isolates from *V. californica* and *V. californica* × *V. vinifera* hybrids were closely related to isolates from *V. vinifera*.

Grapevine leafroll disease is one of the most economically damaging and widespread diseases of *Vitis vinifera* throughout the world. To date, 11 different phloem-limited viruses, identified as Grapevine leafroll-associated virus (GLRaV)-1 to -7, -9, -10, -11, and Carnelian have been isolated from diseased grapevines and characterized (31). All of the GLRaVs can be graft transmitted; GLRaV-1, -3, -5, and -9 are also transmitted by several species of mealybugs (Hemiptera: Pseudococcidae) and soft-scale insects (Hemiptera: Coccidae; 32). The most common vectors in northern California vineyards are grape mealybug (*Pseudococcus maritimus* Ehrhorn), obscure mealybug (*P. viburni* Signoret), longtailed mealybug (*P. longispinus* Targioni-Tozzetti), and citrus mealybug (*Planococcus citri* Risso; 23).

Although the incidence of leafroll disease due to graft transmission has been reduced in propagative material through the use of certified clean planting stock, disease incidence associated with insect transmission is still occurring in many grape-growing regions. Disease distribution patterns in Spain (11,12), the United States (24), and New Zealand (13) vineyards indicate two common patterns: (i) two or more infected vines directly adjacent to each other in the same row and (ii) a focus of infected vines at the edges of a vineyard with a gradient of diminishing incidence toward the middle or other side of the vineyard. This latter pattern suggests that leafroll disease has been introduced into the vineyard from an established external source on one side of the vineyard. In most cases, the number of infected vines in these gradients increases in the opposite direction of an adjacent older, infected vineyard. These vineyards probably serve as the source of the disease where mealybugs acquire the virus and then are spread to neighboring vineyards by some combination of wind, laborers, or machinery (13,30). The introduction of leafroll disease from outside sources is particularly worrisome because it is difficult to control and has the potential to compromise the benefits of clean plant programs.

Although older infected vineyards are an obvious disease source, it is possible that other, as-yet-unidentified outside sources also exist. At one site, the disease distribution gradients of Cabaleiro

and Segura (11) indicated a positive association with a stand of *Eucalyptus* spp. in addition to neighboring infected vineyards, suggesting that this non-*Vitis* spp. may be an alternate host for GLRaV-3. Support for non-*Vitis* hosts also comes from the work of Mikona and Jelkmann (34), who demonstrated that GLRaV-7 can be transmitted from an Albanian grapevine accession to *Tetragonia expansa* (New Zealand spinach) by the parasitic dodder *Cuscuta reflexa*, and to *Nicotiana occidentalis* (tobacco) by *C. europea*. In addition, Grapevine virus A (GVA), a virus often found in mixed infections with the GLRaVs, has been transmitted under experimental conditions to *Nicotiana* plants using mealybugs (17,20), and Grapevine virus B (GVB) has been acquired by *Cuscuta* spp. (dodder; 21) and *Nicotiana* spp. (20).

Alternate outside sources could also include endemic *Vitis* spp. *V. aestivalis* is a host to two new viruses in taxa whose members are not previously known to infect *Vitis* spp.: a multipartite double-stranded RNA virus related to Rice ragged stunt virus (*Reoviridae* family) and a virus with similarities to Pea enation mosaic virus 1 (*Luteoviridae* family; 22). *V. californica*, a common endemic California grape species, is an important alternate host for two nonviral grape pathogens: *Daktulosphaira vitifoliae*, the aphid-like insect that causes grape phylloxera (25), and *Xylella fastidiosa*, the bacterium that causes Pierce's Disease (5,42).

In an effort to identify possible alternate hosts for the GLRaVs and vitiviruses, we surveyed numerous *Vitis* and non-*Vitis* spp. within and surrounding nine vineyards in Napa County, CA, with vines showing typical leafroll disease symptoms. We also identified three riparian areas in Napa and Yolo Counties that were not near vineyards but contained a number of endemic *Vitis* spp. with red leaves.

Materials and Methods

Vineyards and sample collection. In October and November 2008 and 2009, seven and two vineyards, respectively, were selected in Napa County, CA that had *V. vinifera* with red leaf symptoms typical of leafroll disease adjacent to areas with noncultivated *Vitis* and non-*Vitis* spp. Three riparian areas were also chosen that were not adjacent to vineyards but had a number of noncultivated *Vitis* plants with red leaves. Outside the vineyard, *Vitis* spp. and the most common woody non-*Vitis* spp. that were present within a 25-m² area adjacent to symptomatic *V. vinifera* blocks were collected (Table 1). Within each vineyard, at least three vines from a block adjacent to noncultivated *Vitis* or non-*Vitis* spp. were collected. Composite samples of five to six canes per vine were collected and

Corresponding author: D. A. Golino, E-mail: dagolino@ucdavis.edu

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stored at 4°C. The location of each sample was geospatially referenced and vines were tagged for future reference. Several vines collected in fall 2008 were resampled in fall 2009 to confirm the previous year's results.

Herbaceous plants were collected in April and May 2009 by establishing three 1-m² plots located within 10 m of the symptomatic *V. vinifera* collected the previous fall. Within each plot, three replicate samples of all the dicotyledonous species were collected and pooled (Table 1). Like species were also pooled between plots due to the high number of different species. Samples were stored at 4°C for no longer than 2 days before they were homogenized and extracted for total RNA.

Positive and negative controls for GLRaV-1 to -5, -9, GVA, GVB, and GVD were maintained in *V. vinifera* grown in the greenhouse or the field.

Virus detection. *Vitis* and woody samples collected in fall 2008 were assayed for GLRaV-1 to -5, -9, GVA, GVB, and GVD by both conventional and quantitative (q) reverse-transcription polymerase chain reaction (RT-PCR) (TaqMan; Applied Biosystems Inc., Foster City, CA). Only qRT-PCR was used to detect these same viruses in herbaceous samples collected in spring 2009 and *Vitis* and non-*Vitis* samples collected in fall 2009. Pooled cambial scrapings from woody stems or petioles from herbaceous plants (200 mg) were homogenized in 1:10 guanidine buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate [pH 5.0], 0.025 M EDTA, and 2.5% [wt/vol] PVP-40) using a Bioreba Homex 6

grinder (STA Labs, Longmont, CO). Total RNA was extracted from the lysate of homogenized material using the RNeasy Mini Plant kit and Qiashredder columns (Qiagen, Valencia, CA). Total purified RNA (2 µl) was used in a 25-µl final volume for one-step RT-PCR (37,41) and 2 µl of the same RNA was used in a 12-µl final volume for qRT-PCR (37,39). The RT-PCR primers (Invitrogen Life Technologies, Carlsbad, CA) and qRT-PCR primers/TaqMan probes (MWG Inc., Biotech, NC) for GLRaV-1 to -5, -9, GVA, GVB, and GVD are listed in Table 2. An 18S rRNA qRT-PCR assay (36) was used to verify that the total RNA samples could be amplified. qRT-PCR assays were run in duplicate or triplicate, and data were analyzed quantitatively by measuring the cycle threshold (*C_T*) in a Microsoft Excel format, and graphically by an amplification plot.

Viral DNA sequences. Total RNA from the samples positive by qRT-PCR was reamplified using RT-PCR. RT-PCR products were eluted from agarose gels using the ZymoClean Gel DNA Recovery Kit (ZymoResearch, Orange, CA) and submitted for direct sequencing at the College of Biological Sciences, University of California (UC) DNA Sequencing Facility (UC Davis). Nucleic acid sequences were compared using BLAST (4) from the National Center for Biotechnology Information, and aligned and edited using CLUSTALX (45) version 2.0.10. The phylogenetic trees were obtained with PHYLIP (18) using the neighbor-joining method and 1,000 bootstrap repetitions as a confidence test of groupings. Branches with less than 70% support were not labeled. The Gen-

Table 1. List of non-*Vitis* spp. assayed for Grapevine leafroll-associated virus-1 to -5 and -9 and Grapevine virus A, B, and D^a

Family	Species	Common name
Aceraceae	<i>Acer macrophyllum</i>	Bigleaf maple
Anacardiaceae	<i>Toxicodendron diversilobum</i>	Poison oak
Apiaceae	<i>Anthriscus caucalis</i>	Bur chervil
	<i>Conium maculatum</i>	Poison hemlock
	<i>Daucus carota</i>	Wild carrot
	<i>Torilis</i> spp.	Torilis
Apocynaceae	<i>Vinca</i> spp.	Vinca
Asteraceae	<i>Ambrosia psilostachya</i>	Western ragweed
	<i>Baccharis pilularis</i>	Coyote bush
	<i>Calendula arvensis</i>	Field marigold
	<i>Carduus pycnocephalus</i>	Italian thistle
	<i>Chamomilla suaveolens</i>	Pineapple weed
	<i>Hypochaeris glabra</i>	Smooth cats ear
	<i>Lactuca saligna</i>	Willow leaf lettuce
	<i>Lactuca serriola</i>	Prickly lettuce
	<i>Picris echanoides</i>	Bristly ox-tongue
	<i>Rhagadiolus stellatus</i>	Endive daisy
	<i>Senecio vulgaris</i>	Groundsel
	<i>Sonchus oleraceus</i>	Annual sowthistle
Boraginaceae	<i>Amsinckia</i> spp.	Fiddleneck
Brassicaceae	<i>Brassica rapa</i>	Mustard
	<i>Cardamine hirsuta</i>	Hairy bittercress
	<i>Hirshfeldia incana</i>	Shortpod mustard
	<i>Lepidium nitidum</i>	Pepperweed
Calycanthaceae	<i>Calycanthus occidentalis</i>	Spicebush
Caprifoliaceae	<i>Sambucus</i> spp.	Elderberry
	<i>Symphoricarpos</i> spp.	Snowberry
Convolvulaceae	<i>Convolvulus arvensis</i>	Bindweed
Cucurbitaceae	<i>Marah</i> spp.	Wild cucumber
Fabaceae	<i>Acacia dealbata</i>	Silver wattle
	<i>Genista monspessulanus</i>	French broom
	<i>Lupinus</i> spp.	Lupine
	<i>Medicago arabica</i>	Spotted burclover
	<i>Medicago polymorpha</i>	California burclover
	<i>Trifolium hirtum</i>	Rose clover
	<i>Trifolium</i> spp.	Clover
	<i>Trifolium subterraneum</i>	Subterranean clover
	<i>Vicia</i> spp.	Vetch
	<i>Vicia villosa</i>	Hairy vetch
	<i>Quercus agrifolia</i>	Coast live oak
	<i>Quercus lobata</i>	Valley oak

(continued on next page)

^a At each location, three replicate samples of all dicotyledonous species were collected from each of three plots. Like species were pooled between samples and plots.

Bank accession numbers for the sequences reported in this article are HQ185602 to HQ185618 and HQ676994 to HQ677008, which are also listed in Table 3.

Vitis genotype. The noncultivated *Vitis* samples from outside the vineyards were analyzed at 18 simple sequence repeat (SSR) DNA markers: VVMD5, VVMD6, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD31, VVMD32, VVMD34, and VVMD36 (9,10); VrZAG62, VrZAG79, VrZAG83, and VrZAG93 (43); and VVS2 and VVS29 (44). Genomic DNA was extracted from dried leaf or cambial tissue using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. PCR, fragment separation, and sizing of amplified fragments were performed according to Bautista et al. (6). The resulting DNA profiles were compared with over 1,200 unique grape DNA profiles, including *V. vinifera* cultivars and rootstock genotypes, to identify escapes and to determine the parent cultivars of apparent first-generation hybrids. To identify the species background of the remaining samples, a Bayesian approach (formula 9; 40) was used to calculate the probability of observing each unique profile from this study and those of five diverse *V. vinifera* cultivars based on allele frequencies from a population of 200 random *V. vinifera* cultivars. The results were expressed as the inverse of these probabilities divided by the average of the probabilities of the five *V. vinifera* profiles.

Results

Plant survey. In fall and spring 2008 and 2009, 303 plant samples were collected from in and around nine vineyards (sites 1 to 9) located in a 176-km² area in Napa Valley; two riparian areas (sites

10 to 11) characterized by willows, Fremont cottonwoods, and noncultivated *Vitis* spp., located approximately 15 and 12 km northeast of Napa Valley; and the UC Davis Arboretum (site 12), located 80 km southeast of Napa Valley. The samples included 94 noncultivated *Vitis* plants, 145 non-*Vitis* plants representing 83 species (Table 1), and 64 cultivated *V. vinifera* plants from within vineyards. The 64 *V. vinifera* cultivars included 'Syrah', 'Pinot noir', 'Petite Verdot', 'Merlot', 'Cabernet Sauvignon', and 'Cabernet franc'.

Noncultivated *Vitis* genotypes. Leaf morphology indicated that the majority of the 94 noncultivated *Vitis* plants were *V. californica*. This was also confirmed by genetic analyses. DNA fingerprinting indicated 48 unique multilocus genotypes; 4 of these were known scion or rootstock cultivars and were excluded from further analyses. Within the remaining 44 genotypes, we identified a self-seedling of Cabernet franc, and six first-generation *V. vinifera* hybrids with Cabernet Sauvignon, 'Malbec', Merlot, and 'Alicante Bouschet' as parents. Species designations were assigned to the 38 remaining unique genotypes by calculating the probability of a sample being *V. vinifera*, not *V. californica*, because there is no database of *V. californica* profiles. In this manner, decreasing *V. vinifera* contribution was illustrated (Fig. 1). Probability analysis indicated that the unique *Vitis* genotypes formed three groups: *V. vinifera*, which included the Cabernet franc self-seedling; *V. vinifera* hybrids, which included the six first-generation hybrids; and non-*V. vinifera* (Fig. 1). Two samples in the non-*V. vinifera* group had 'St. George', a *V. rupestris* rootstock, as a parent; another sample was part rootstock. The remaining 36 profiles had less than

Table 1. (continued from preceding page)

Family	Species	Common Name
Geraniaceae	<i>Erodium botrys</i>	Broad-leaved filaree
	<i>Erodium cicutarium</i>	Red-stemmed filaree
	<i>Erodium moschatum</i>	White-stemmed filaree
	<i>Geranium dissectum</i>	Cutleaf geranium
	<i>Geranium molle</i>	Dovefoot geranium
	<i>Geranium purpureum</i>	Little Robin
Hamamelidaceae	<i>Liquidamber styraciflua</i>	Sweetgum
Juglandaceae	<i>Juglans nigra</i>	Black walnut
Lamiaceae	<i>Lamium purpureum</i>	Purple deadnettle
Lamiaceae	<i>Melissa officinalis</i>	Lemon balm
	<i>Stachys ajugoides rigida</i>	Hedge nettle
Lauraceae	<i>Umbellularia californica</i>	CA Bay laurel
Malvaceae	<i>Malva</i> spp.	Cheeseweed
Onagraceae	<i>Epilobium branchycarpum</i>	Tall annual willowherb
Papaveraceae	<i>Eschscholzia californica</i>	California poppy
Plantaginaceae	<i>Plantago lanceolata</i>	Buckhorn plantain
Poaceae	<i>Avena fatua</i>	Oat
	<i>Bromus diandrus</i>	Ripgut brome
	<i>Hordeum</i> spp.	Foxtail
	<i>Lolium multiflorum</i>	Ryegrass
	<i>Vulpia myuros</i>	Rattail fescue
Polygonaceae	<i>Rumex acetosella</i>	Red sorrel
	<i>Rumex crispis</i>	Curly dock
	<i>Rumex obtusifolia</i>	Broad leaved dock
Portulacaceae	<i>Calandrinia ciliata</i>	Red maids
	<i>Claytonia perfoliata</i>	Miners lettuce
Ranunculaceae	<i>Ranunculus muricatus</i>	Roughseed buttercup
Rosaceae	<i>Heteromeles arbutifolia</i>	Toyon
	<i>Prunus cerasifera</i>	Prunus
	<i>Prunus</i> spp.	Ornamental plum
	<i>Prunus dulcis</i>	Almond
	<i>Rosa californica</i>	California rose
	<i>Rubus armeniacus</i>	Himalayan blackberry
Rubiaceae	<i>Galium aparine</i>	Bedstraw
Salicaceae	<i>Populus</i> spp.	Poplar
	<i>Salix laevigata</i>	Red Willow
	<i>Salix lasiolepis</i>	Arroyo Willow
	<i>Salix</i> spp.	Willow
Scrophulariaceae	<i>Kickxia elatini</i>	Fluvelin
	<i>Scrophularia californica</i>	Scrophularia
Vitaceae	<i>Parthenocissus tricuspidata</i>	Boston ivy, grape ivy
	<i>Parthenocissus quinquefolia</i>	Virginia creeper

10% of alleles in common with rootstock cultivars (*data not shown*) and, therefore, were non-rootstock as well as non-*V. vinifera*. Therefore, we concluded that these vines were primarily *V. californica* and that the *V. vinifera* hybrids were *V. californica* × *V. vinifera*.

RT-PCR assays. The *Vitis* and non-*Vitis* samples collected in fall 2008 were assayed using both conventional RT-PCR and qRT-

PCR. Seventy-five *Vitis* samples had positive C_T values between 17.1 and 35.6 which, for all samples, indicated a nucleic acid amount within a 10^5 -fold difference relative to positive controls. Of these 75 positive samples, 29 were positive by both RT-PCR and qRT-PCR (*data not shown*) while 46 were positive by qRT-PCR but negative by RT-PCR. To confirm that these 46 samples were, in fact, positive, we rescrapped the canes, extracted total RNA, and

Table 2. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative (q)RT-PCR primer and probe nucleotide sequences used in *Grapevine leafroll-associated virus-1* to -5 and -9 and *Grapevine virus A, B, and D* assays

Virus	Assay	Primer	Target gene ^a	Primer sequence (5'–3')	Reference
GLRaV-1	RT-PCR	LR1 HSP70-417 f ^b	hHSP70	GAGCGACTTGCGACTTATCGA	38
	...	LR1 HSP70-737 r ^c		GGTAAACGGGTGTTCTTCAATTCT	...
GLRaV-2	RT-PCR	LR2-L2-f	hHSP70	ATAATTCGGCGTACATCCCCACTT	8
	...	LR2-U2-r	...	GCCCTCCGCGCAACTAATGACAG	...
	...	LR2-CPV ^b	CP	TGGAGTTGATGTCCGACAGC	...
	...	LR2-CPC ^c	...	ACGACCGAACGTTCTAAGTT	...
GLRaV-3	RT-PCR	LC1-f	hHSP70	CGCTAGGGCTGTGGAAGTATT	38
	...	LC2-r	...	GTTGTCCCGGGTACCAGATAT	...
	...	LR3 CP-ORF6-111f	CP	AAAGTACGTAAAGGACGGGACACA	...
	...	LR3 CP-ORF6-722r	...	AGGGTCGCCGTGATGAAG	...
GLRaV-4	RT-PCR	LR4-HSP V	hHSP70	ACATTCTCCACCTTGTGCTTTT	36
	...	LR4- HSP C	...	CATACAAGCGAGTGCAATTACA	...
GLRaV-5	RT-PCR	LR 5-HSP V	hHSP70	AACACTCTGCTTTTCTGCTGGCA	36
	...	LR 5-HSP C	...	TCTCCAGAAGACGGACCAATGTAA	...
GLRaV-9	RT-PCR	LR9-F/f	hHSP70	CGGCATAAGAAAAGATGGCAC	3
	...	LR9-R/r	...	TCATTCCACCTGCTTGAAC	...
GVA	RT-PCR	GVA V1	CP	GACAAATGGCACACTACG	35
	...	GVA C1	...	AAGCCTGACCTAGTCATCTTGG	...
GVB	RT-PCR	GVB V1	CP	GTGCTAAGAACGTCTTCACAGC	35
	...	GVB C1	...	ATCAGCAAACACGCTTGAACCG	...
GVD	RT-PCR	GVD CP 7 V	CP	CTTAGGACGCTCTTCGGGTACA	2
	...	GVD CP 471 C	...	CTGCTCTCCAACCGACGACT	...
GLRaV-1	qRT-PCR	LR1-149f	hHSP70	ACCTGGTTGAACGAGATCGCTT	This study
	...	LR1-149 f2	...	ACTTGGTTGAACGAGATTGCTT	...
	...	LR1-149 f3	...	ACCTGGTTGAATGAGATCGCTT	...
	...	LR1-293r	...	GTAACCGGGTGTCTTCAATTCTCT	...
	...	LR1-225 p2	...	ACGAGATATCAGTGGACGGA	...
GLRaV-2	qRT-PCR	LR2-124f	hHSP70	CGATTCTAACCACATGACTTTTGG	This study
	...	LR2-284r1	...	GGACCGAGTAATGAGGTTTAAACG	...
	...	LR2-284r2	...	GGACTGAGTAGTGTGGTTTAAACG	...
	...	LR2-284r3	...	GGACTGAATAGTGTGGTTTAAACG	...
	...	LR2-214p ^d	...	CGTTGGGTGGGTTGCGATTTCGAG	...
GLRaV-3	qRT-PCR	LR3 new-f	hHSP70	GGGTCAAGTCTAGTTAAGGTCA	This study
	...	LR3new-r1	...	AAAGTGTCCACCACTCTCAGTCC	...
	...	LR3new-r2	...	AAAGTGTCCACCAATCTCAGTCC	...
	...	LR3new-p1	...	TTGCCGAGATATCTA	...
	...	LR3new-p2	...	TTGCCGACATATC	...
GLRaV-4	qRT-PCR	LR4 hsp-85f	hHSP70	ATATACATACCAACCGTTGTGGGTATAA	36
	...	LR4hsp-178r	...	CCCTATAAACTAGCACATCCTTCTCTAGT	...
	...	LR4hsp-120p	...	TGGAACATATACCATTGGGCTTGGTGCT	...
GLRaV-5	qRT-PCR	GLRaV5-26f	hHSP70	AACACTCTGCTTTTCTGCTGGC	36
	...	GLRaV5-188r	...	CTTTTATGTCCCGATAAACGAGTACA	...
	...	GLRaV5-131p	...	CAATAGGTTTGGGGGCTTGTCTAGAAAAGG	...
GLRaV-9	qRT-PCR	LR9-114f	hHSP70	CGGCATAAGAAAAGATGGCAC	36
	...	LR9-196r	...	TCTTTATGTCACGGTAGACCAACAC	...
	...	LR9-136p	...	TATACCATAGGATTGGGAGCTCTGCTGGAGA	...
GVA	qRT-PCR	GVA-77 f1	CP	CGACCGAAATATGTACCTGAATACTC	39
	...	GVA-77 f2	...	CGACCGAACTATGTACCTGAATACTC	...
	...	GVA-192 r1	...	TTTGCTAGCTTTAGGACCTACTATATCTACCT	...
	...	GVA-192 r2	...	CTTGCTAGCCTTAGGTCCTACTATATCTACCT	...
	...	GVA-104 p	...	CTTCGGGTACATCGCCTTGGTCGG	...
GVB	qRT-PCR	GVB-92 f1	CP	CTAGGAGTGCGGCTAAACGAA	39
	...	GVB-92 f2	...	GGAGTGCGGCGCAACGA	...
	...	GVB-95 f3	...	CAAGGAGTGCGGCTAAACGAA	...
	...	GVB-202 r1	...	CCTTAACCTCGTCCTGTGATATGGT	...
	...	GVB-202 r2	...	CCTTCACCTCATCTGTGGATCGTGT	...
	...	GVB-119 p1	...	CTCGTTATGGTCGCTGTACTGTTGTGGTAG	...
	...	GVB-119 p2	...	ACCGTTACGGCCGTTGTTACTGTTGTGGTAG	...
GVD	qRT-PCR	GVD-554 f	CP	AGGTGTATTCAACGCCAGTCG	39
	...	GVD-631 r	...	CCTGCGAGAAATGATGGGTCA	...
	...	GVD-580 p	...	TTGGAGAGCAGCGAGTTGAGATATAAATGGA	...

^a hHSP70 = homologous heat-shock 70 protein gene and CP = coat protein gene.

^b Forward primer orientation.

^c Reverse primer orientation.

^d qRT-PCR probe.

Table 3. Virus, isolate and GenBank accession numbers for the partial coat protein gene sequences of *Grapevine leafroll-associated virus* (GLRaV)-2 and -3 and *Grapevine virus A* (GVA) and *B* (GVB)

Virus, isolate	GenBank accession
GLRaV-2	
LN	FJ786017
SE	EU204909
Rei	DQ314604
Temp	DQ314608
OR-G5b	GU457406
IT	EU204910
Muscat	EF118030
WA-HHC1	EU760847
PMC-083	HM185277
Prim	DQ314601
NA1	DQ314595
GLA-110	HM185278
Shandong	AY842932
IsP	DQ314591
Red2	DQ314603
SL10	DQ911147
Pol1	DQ314599
Arv1	DQ314588
Ner	DQ314598
WA-MCU3	EU760841
Y204	EF118023
Cf	FJ195744
H4	AY697863
MH	EU204911
RI	EU204912
WA-HHA7	EU760844
WA-HHA6	EU760846
RG2	DQ314606
RG1	DQ314605
GRSLaV	AF314061
WA-Chot2	WA-Chot2
BD	DQ286725
PV44	EF012718
PV20	EF012721
PV124	EF012717
<i>V. vinifera</i> 155*	HQ185614
<i>V. vinifera</i> 156*	HQ185615
<i>V. californica</i> 162*	HQ185616
<i>V. californica</i> 163*	HQ185617
<i>V. californica</i> 164*	HQ185618
GLRaV-3	
SS5.2	AJ606347
GR1.1	AJ606342
LN	FJ786016
PL20	GQ352633
Pet1	DQ680141
Dawanhong2	DQ119574
CL664	EU344895
MT48	AJ606351
NY1	AF037268
CL765	EU344896
CL766	EU344893
CL817	EU344894
USA	AJ606340
AUSG5.2	AJ606339
IS2	HM059034
GP18	EU259806
<i>V. californica</i> × <i>V. vinifera</i> 98*	HQ185602
<i>V. californica</i> × <i>V. vinifera</i> 99*	HQ185603
<i>V. californica</i> × <i>V. vinifera</i> 125*	HQ185604
<i>V. rupestris</i> 126*	HQ185609
<i>V. californica</i> × <i>V. vinifera</i> 127*	HQ185610
<i>V. vinifera</i> × <i>V. rupestris</i> 128*	HQ185611
Merlot 149 ^a	HQ185608
Merlot 150 ^a	HQ185607
Chenin Blanc escape 155 ^a	HQ185606
Cabernet Sauvignon 159 ^a	HQ185605
Cabernet Sauvignon 160 ^a	HQ185612
Cabernet Sauvignon 161 ^a	HQ185613

(continued in next column)

^a Nucleotide sequences from this study which are indicated by *Vitis* spp. or *V. vinifera* cultivar and sample number.

Table 3. (continued from preceding column)

Virus, isolate	GenBank accession
<i>V. californica</i> 113 ^a	HQ676994
<i>V. californica</i> 114 ^a	HQ676995
<i>V. californica</i> 162 ^a	HQ676996
<i>V. californica</i> 163 ^a	HQ676997
<i>V. californica</i> 164 ^a	HQ676998
GVA	
Merlot 97 ^a	HQ676999
Chenin Blanc 155 ^a	HQ677000
<i>V. californica</i> 162 ^a	HQ677001
<i>V. californica</i> 163 ^a	HQ677002
<i>V. californica</i> 164 ^a	HQ677003
GVB	
Merlot 119 ^a	HQ677004
Merlot 120 ^a	HQ677005
<i>V. californica</i> 162 ^a	HQ677006
<i>V. californica</i> 163 ^a	HQ677007
<i>V. californica</i> 164 ^a	HQ677008

Fig. 1. Unique *Vitis* genotypes ($n = 44$) calculated using a Bayesian approach. Each circle represents a unique genotype; solid circles represent genotypes that are positive for *Grapevine leafroll associated virus* 2 or 3 or *Grapevine virus A* or *B*. The *Vitis vinifera* group includes the *V. vinifera* self-seedling and the five diverse *V. vinifera* cultivars ('Pinot Noir', 'Zinfandel', 'Cabernet Sauvignon', 'Mission', and 'Alicante Bouchet') used in comparison. For each of the 44 unique genotypes, the value shown is the inverse $\log_{10}$ of the probability of the genotypes being *V. vinifera* divided by the average probability associated with the five diverse *V. vinifera* profiles.

repeated the qRT-PCR assay. All of these samples had C_T values less than 36, confirming our initial results and indicating that the qRT-PCR system was more sensitive than RT-PCR for detecting the viruses in this study. Based on our results with these *Vitis* and non-*Vitis* samples, only qRT-PCR was used to assay the herbaceous non-*Vitis* samples collected in spring 2009 and the *Vitis* and non-*Vitis* samples collected in fall 2009. Positive C_T values for these samples were between 16.7 and 35.9.

The 18S rRNA qRT-PCR positive C_T values were between 7.8 and 13.8 for all but two of the *Vitis* and non-*Vitis* samples, verifying the high quality of the majority of the total RNA. No positive C_T values were obtained in assays of total RNA from healthy vines except in the 18S assay.

Virus incidence. In the areas adjacent to vineyards (sites 1 to 9), 3 of the 45 *V. californica* samples were positive for GLRaV-3; these occurred at site 3 (Table 4). Of the 15 *V. californica* × *V. vinifera* samples, 7 were positive; these occurred at sites 2, 3, and 4 (Table 4). Five of these seven samples were positive for GLRaV-3 only (sites 2 and 3) and two samples were positive for both GLRaV-3 and GVA (site 4). The *V. vinifera* self-seedling at site 9 was positive for all four viruses, and the scion or rootstock escapes at sites 1, 2, 8, or 9 were positive for GLRaV-2, -3, GVA, or GVB (Table 4). The 145 non-*Vitis* samples tested negative for all viruses under investigation (*data not shown*).

Within vineyards, 39 of 64 cultivated *V. vinifera* samples were positive for +++GLRaV-2, GLRaV-3, GVA, or GVB in single or mixed infections (Table 4). We did not detect any virus-positive

samples at sites 2, 4, and 5 despite the presence of leafroll disease symptoms.

In riparian areas not near vineyards, 3 of the 21 *V. californica* samples were positive for GLRaV-2, -3, GVA, and GVB; these occurred at sites 10 and 11 (Table 4). The three *V. californica* × *V. vinifera* hybrids at the UC Davis Arboretum were positive for GLRaV-2 and GVB (Table 4). We found one *V. californica* × *V. vinifera* hybrid at site 10 that was positive for GVA only (Table 4).

No *Vitis* or non-*Vitis* samples were positive for GLRaV-1, -4, -5, -9, or GVD.

Viral sequences. We obtained partial coat protein (CP) gene sequence data for 5 GLRaV-2, 17 GLRaV-3, 5 GVA, and 5 GVB isolates from noncultivated *Vitis* and adjacent *V. vinifera* plants. The GLRaV-2 RT-PCR products were 88 to 96% identical at the nucleotide level to GLRaV-2 partial CP gene sequences in GenBank; GLRaV-3 products were 88 to 100% identical to GLRaV-3 partial CP gene sequences; GVA products were 91 to 93% identical to GVA partial CP gene sequences; and GVB products were 91 to 97% identical to GVB partial CP gene sequences. We were not able to amplify and obtain nucleotide sequence data for GLRaV-2 from *V. californica* × *V. vinifera* hybrids even though we were able to detect qRT-PCR products.

Phylogenetic analysis of GLRaV-2 partial CP gene sequences revealed that our *V. californica* and *V. vinifera* sequences were closely related to each other and to other members of the PN lineage (27,33; Fig. 2A). However, the relationship of members within the PN clade could not be determined due to low bootstrap support for the subgroups.

The 17 GLRaV-3 partial CP gene sequences formed two groups (Fig. 2B). The first group contained sequences for *V. vinifera* Cabernet Sauvignon, the 'Chenin Blanc' escape, and two first-generation *V. californica* hybrids with Cabernet Sauvignon as the *V. vinifera* parent (Fig. 2B). The second group contained sequences for *V. vinifera* Merlot, two escape rootstocks, *V. rupestris* (St. George) and *V. vinifera* × *V. rupestris* (AXR), two first-generation *V. californica* hybrids with Merlot as the *V. vinifera* parent, and five

V. californica isolates (Fig. 2B). Each of the escape rootstock isolates formed a strongly supported group with a wild *Vitis* isolate (Fig. 2B). The remaining relationships within these groups could not be determined due to low bootstrap support.

Discussion

We identified GVA- or GLRaV-3-positive *V. californica* or *V. californica* × *V. vinifera* hybrids in the areas adjacent to three vineyards. The remaining six vineyards did not have any virus-positive *V. californica* or hybrids, even though the majority of these sites had relatively large populations of these *Vitis* spp. This suggests that GLRaV-3 and GVA incidence was not widespread in *V. californica* and hybrids even though these grapevine species were common in our sampling areas. Localized incidence was also supported by the close proximity of sites 2 to 4 to each other (0.8 to 1.6 km) compared with the other sites.

Phylogenetic analysis of GLRaV-3 partial CP gene sequences indicated two points of interest. First, the GLRaV-3 isolates from *V. californica* and the *V. californica* × *V. vinifera* first-generation hybrids were closely related to GLRaV-3 isolates from *V. vinifera*. This suggests that these wild isolates probably do not represent a major source of diversity that could lead to major epidemics in *V. vinifera* (28). It is important to remember, however, that our sequence data is from RT-PCR products, not clones, and, therefore, represents the dominant population. More diverse sequences may be present in low abundance. Second, the GLRaV-3 isolates from hybrid *Vitis* plants in riparian areas formed two distinct groups based on their respective *V. vinifera* parents, Cabernet Sauvignon and Merlot. Phylogenetic groups distinguished by cultivars was also supported by the *V. vinifera* isolate sequences from this study. Both Cabernet Sauvignon and Merlot were located at site 9 yet were infected by different GLRaV-3 strains. This is similar to the results of Engel et al. (16), who found different GLRaV-3 strains in Merlot versus 'Chardonnay' within one vineyard, but contrasts with Turturo et al. (46), who found similar GLRaV-3 strains in different cultivars. Although these contrasting results within *V.*

Table 4. Incidence of Grapevine leafroll associated virus (GLRaV)-2 and -3 and Grapevine virus A (GVA) and B (GVB)

Virus, <i>Vitis</i> type ^b	Incidence (positive/total) ^a											
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10	Site 11	Arboretum
GLRaV-2												
<i>V. californica</i>	0/7	—	0/3	—	0/6	—	0/11	0/18	—	2/16	1/5	—
Hybrids	—	0/3	0/2	0/2	0/4	—	0/2	0/2	—	0/1	—	3/3
<i>V. vinifera</i> self-seedling	—	—	—	—	—	—	—	—	1/1	—	—	—
Rootstock hybrids	0/1	—	—	—	—	—	—	0/1	—	—	0/1	—
Escapes	0/1	0/2	—	—	—	—	—	1/1	1/1	—	—	—
<i>V. vinifera</i>	6/6	0/5	4/4	0/11	0/3	9/9	1/5	7/13	0/8	—	—	—
GLRaV-3												
<i>V. californica</i>	0/7	—	3/3	—	0/6	—	0/11	0/18	—	2/16	1/5	—
Hybrids	—	3/3	2/2	2/2	0/4	—	0/2	0/2	—	0/1	—	0/3
<i>V. vinifera</i> self-seedling	—	—	—	—	—	—	—	—	1/1	—	—	—
Rootstock hybrids	0/1	—	—	—	—	—	—	0/1	—	—	0/1	—
Escapes	0/1	2/2	—	—	—	—	—	0/1	1/1	—	—	—
<i>V. vinifera</i>	1/6	0/5	0/4	0/11	0/3	2/9	5/5	0/13	8/8	—	—	—
GVA												
<i>V. californica</i>	0/7	—	0/3	—	0/6	—	0/11	0/18	—	2/16	1/5	—
Hybrids	—	0/3	0/2	2/2	0/4	—	0/2	0/2	—	1/1	—	0/3
<i>V. vinifera</i> self-seedling	—	—	—	—	—	—	—	—	1/1	—	—	—
Rootstock hybrids	0/1	—	—	—	—	—	—	0/1	—	—	0/1	—
Escapes	0/1	0/2	—	—	—	—	—	0/1	1/1	—	—	—
<i>V. vinifera</i>	3/6	0/5	0/4	0/11	0/3	3/9	0/5	0/13	2/8	—	—	—
GVB												
<i>V. californica</i>	0/7	—	0/3	—	0/6	—	0/11	0/18	—	2/16	1/5	—
Hybrids	—	0/3	0/2	0/2	0/4	—	0/2	0/2	—	0/1	—	3/3
<i>V. vinifera</i> self-seedling	—	—	—	—	—	—	—	—	1/1	—	—	—
Rootstock hybrids	0/1	—	—	—	—	—	—	0/1	—	—	0/1	—
Escapes	0/1	0/2	—	—	—	—	—	0/1	1/1	—	—	—
<i>V. vinifera</i>	5/6	0/5	3/4	0/11	0/3	3/9	2/5	0/13	0/8	—	—	—

^a Number of samples that were positive at a given site for a given virus/number of samples at each site. Bold numbers indicate sites with positive samples. A positive was recorded for each virus in a mixed infection; — indicates that no *Vitis* spp. were present.

^b Hybrids = *Vitis californica* × *V. vinifera* hybrids and Escapes = *V. vinifera* 'Chenin Blanc', *V. rupestris*, and *V. vinifera* × *V. rupestris*.

vinifera may be explained by the presence of similar or distinct GLRaV-3 strains in the source material which are then transmitted to the scion during propagation steps, the possible origin of the distinct GLRaV-3 isolates in the first-generation hybrids with different *V. vinifera* parents is more difficult to establish.

It was not clear how widespread GLRaV-3 and GVA were within these three sites because our sample numbers were too low to estimate incidence with a known confidence level. It is noteworthy, however, that 100% of these samples were positive for at least GLRaV-3. This suggests that, once GLRaV-3 is present in an area, it may spread, presumably via mealybugs (23). The extent and rate of GLRaV-3 spread within *V. californica* and hybrid populations would be influenced by similar variables, including mealybug population numbers (13), distance between vines (26,30), and temperature and wind speed (30), that appear to influence the spread of these viruses within vineyards. Because grapevines in noncultivated areas are often widely dispersed, vine-to-vine spread by walking infectious crawlers is less likely (26,30). Wind dispersal of infectious crawlers, however, could play an important role in the vine-to-vine spread of GLRaV-3. Wind dispersal ranges of 3 to 8 m have been observed for grape mealybug crawlers in Washington (26) and 5 to 15 m for mealybug crawlers in New Zealand (30). Although these distances appear small, the distance between most of our samples was within this range.

The presence and spread of GVA or GLRaV-3 in *V. californica* or hybrids obviously requires the presence of a feeding insect vector. In this study, we assumed this was one of the mealybug species common within northern California vineyards that are also known vectors (23). The grapevine mealybug is native to North America, and the obscure and long-tailed mealybugs have probably been present in northern California for nearly a hundred years (14). In addition, the long-tailed mealybug has been observed on the noncultivated grape species *V. capensis* in San Diego County (7).

Although our data establish that *V. californica* and *V. californica* hybrids are potential GLRaV-3 and GVA reservoirs, there was no evidence that these two viruses are moving between uncultivated

areas and vineyards. A significantly positive correlation between virus incidence outside and within vineyards would be an indirect indication of virus movement but would require more extensive sampling. It was interesting to note, though, that sites 2 to 4, the three vineyards with GVA- or GLRaV-3-positive *Vitis* spp. outside vineyards, did not have GVA- or GLRaV-3-positive *V. vinifera* within. It was possible, however, that previous vineyard plantings, not those surveyed, were infected with GLRaV-3 or GVA. Likewise, five of the six vineyards with *V. vinifera* positive for GLRaV-2, -3, GVA, or GVB did not have adjacent virus-positive *V. californica* or hybrids, despite the fact that many of these vines were within several meters of virus-positive *V. vinifera*. In both scenarios, however, the possibility exists that the viruses may have been present but not detected, especially if their incidence was low.

Two additional viruses, GLRaV-2 and GVB, were detected in the *V. californica* and *V. californica* × *V. vinifera* hybrid samples collected within the riparian areas not adjacent to vineyards. These areas are beyond the air-dispersal range of mealybugs, and dispersal by humans is less likely than in areas immediately adjacent to vineyards. The presence of GLRaV-2, -3, GVA, and GVB in more remote areas suggests that they could be endemic to northern California. Phylogenetic analysis of GLRaV-2, GLRaV-3, GVA, and GVB partial CP gene sequences, however, indicated that all of these *V. californica* isolates were closely related to their respective *V. vinifera* isolates. This close relationship suggested that these *V. californica* isolates were probably introduced into riparian areas relatively recently via infected *V. vinifera* vines.

Three grapevines at the UC Davis Arboretum, the third riparian area, were positive for GLRaV-2 and GVB. The DNA fingerprinting results indicated that these vines, known as 'Roger's Red', were a first-generation cross between *V. californica* and the *V. vinifera* teinturier variety, Alicante Bouschet (15). Roger's Red was originally collected from the wild due to its red fall leaf color, a trait associated with the presence of GLRaV-2 (32). The origin of the GLRaV-2 and GVB infections in the Roger's Red samples cannot be determined but it seems likely, given the presence of these

Fig. 2. Phylogenetic trees constructed from partial coat protein gene sequences of **A**, Grapevine leafroll-associated virus 2 (GLRaV-2) and **B**, Grapevine leafroll-associated virus 3 (GLRaV-3) isolates by the neighbor-joining method with 1,000 bootstrap replicates. Branch length represents phylogenetic distances determined with distance matrices of nucleotide sequences. Numbers above critical branches are significant bootstrap values (>70%). The six GLRaV-2 lineages are indicated (27,33). The two GLRaV-3 groups are denoted by wild *Vitis* genotypes. Representative isolates are indicated by name as well as the country of origin for each sequence accession. GLRaV isolates sequenced in this study are indicated by *Vitis* spp. or *Vitis vinifera* cultivar, sample number, and an asterisk.

two viruses in riparian areas, that the original *V. californica* vine was already infected. These two viruses would have then been transmitted through subsequent propagation steps.

The presence of GLRaV-2 in *V. californica*, *V. californica* hybrids, and a *V. vinifera* self-seedling indicates there may be an unidentified natural vector for this virus. GLRaV-2 is the only leafroll-associated virus included in the genus *Closterovirus* whose other members are transmitted semipersistently by aphids (1,48). No GLRaV-2 aphid or non-aphid vectors have been identified, however, and spread within vineyards has not been observed (27,29). This may be partly due to the fact that the native grapevine aphid, *Aphis illinoisensis*, is not typically considered to be a vineyard pest as long as vines are healthy and insecticides have not significantly reduced the population of natural predators. *A. illinoisensis*, however, could be much more abundant in riparian areas. Several studies have demonstrated that natural zones of overlap and hybridization between plant species support large populations of pests such as the leaf-galling aphid (19,47).

None of the 83 non-*Vitis* plant species tested within and adjacent to vineyards were positive for the viruses included in this study. However, the 18S assay indicated that the total RNA extracts could be amplified in all cases except two, which were excluded from further analyses. Still, there is a possibility that we missed positives in our sampling. The sampling strategy used in this study allowed screening of a relatively large number of different species at each site but the number of samples collected per species was low. Pooling samples to increase the chance of finding positives may have also led to underestimating virus incidence by diluting virus titer below our detection limit.

Once leafroll disease is introduced into clean vineyards, annual spread rates between 8 to 12% have been observed (11–13,24). For a long-term crop such as grapevines, a vineyard can be expected to be fully infected by the time it reaches full productivity. Although we don't yet have any direct or indirect evidence that *V. californica* or *V. californica* hybrids are significant virus reservoirs, there is a need to address questions related to GLRaV-2, -3, GVA, and GVB presence and movement within native *Vitis* populations and between native populations and vineyards. Future studies will focus on more accurately estimating GLRaV-3 and GVA incidence in wild *Vitis* spp. and adjacent *V. vinifera* in vineyards.

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