Limited Genetic Variability Among American Isolates of *Grapevine virus E* from *Vitis* spp.

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Abstract

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A survey for the presence of *Grapevine virus E* (GVE, genus *Vitivirus*, family *Betaflexiviridae*) in vineyards in New York and California was conducted using macroarray hybridization or reverse-transcription polymerase chain reaction (RT-PCR) assays. In New York, GVE was detected in 10 of 46 vines of *Vitis labrusca*, one *V. riparia*, and one *Vitis* hybrid. All GVE-infected New York vines were coinfected with *Grapevine leafroll-associated virus-3*. In California, GVE was detected in 8 of 417 vines of *V. vinifera*. All GVE-infected California vines were also coinfected by one of the leafroll-associated viruses and other vitiviruses. In order to assess the genetic diversity among GVE isolates, a viral cDNA was amplified by RT-PCR, and a 675-nucleotide region that included the 3′ terminus of the coat protein gene, a short intergenic region, and the 5′ terminus of the putative nucleic acid binding protein gene was sequenced. All 20 GVE isolates sequenced in this study were very closely related, with >98% nucleotide identity to the SA94 isolate from South Africa. These findings confirm the presence of GVE in major grape-growing regions of the United States and indicate a very low level of genetic diversity.

Grapevine production is significantly compromised by virus infections, with resulting reductions in the quantity and quality of the crop (Komar et al. 2007; Martelli 2014). To date, 67 viruses have been reported to infect grapevines (Al Rwahnih et al. 2015; Maliogka et al. 2015; Martelli 2014). Four virus-associated disease complexes are of particular concern worldwide: (i) leafroll, (ii) infectious degeneration or decline, (iii) rugose wood, and (iv) fleck. The viruses associated with these diseases are, respectively: members of the genera *Ampelovirus, Closterovirus*, and *Velarivirus* in the family *Closteroviridae* (leafroll viruses) (Maree et al. 2013; Martelli et al. 2012); members of the genus *Nepovirus* (nematode-transmitted viruses) and *Strawberry latent ringspot virus* in the family *Secoviridae* (Martelli and Boudon-Padieu 2006); members of the genera *Vitivirus* and *Foveavirus* (rugose wood-associated viruses) in the family *Betaflexiviridae* (Rosa et al. 2011); and members of the genus *Maculavirus,* family *Tymoviridae* (fleck virus).

Viruses of the genus *Vitivirus* have gained attention in recent years, and there are five recognized vitiviruses associated with grapevine: *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D*, *Grapevine virus E* (GVE), and *Grapevine virus F* (du Preez et al. 2011; Martelli 2014). GVE was first reported associated with rugose wood symptoms in a Japanese grape cultivar in 2008 (Nakaune et al. 2008). Following this report, two new isolates were described along with their full-length sequences, one from South Africa (Coetzee et al. 2010b) and the other from the United States (Alabi et al. 2013). More recently, GVE was detected infecting grapevine in China (Fan et al. 2013) and several grapevine species in New York, including *Vitis labrusca* 'Concord' (Thompson et al. 2014). Although information about Concord-infecting viruses is limited, there are reports of many of the viruses associated with the major groups of grape diseases being detected in Concord vines (Bahder et al. 2013; Ramsdell et al. 1983; Soule et al. 2006; Thompson et al. 2014; Uyemoto et al. 1977). Based on our earlier findings and the small number of reports of GVE, a limited survey was initiated with the objectives of (i) assessing the occurrence of GVE in vines in New York and California and (ii) obtaining an estimate of the genetic diversity for this virus in North America. Because preliminary testing of Concord vines in New York had shown *Grapevine leafroll associated virus-3* (GLRaV-3) to be present, the testing for this virus was an additional objective.

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Materials and Methods

Sampling of grapevines. To determine the occurrence of GVE in North American vines, collections were made from six sites or regions (Table 1). In New York, samples of canes were taken from dormant vines without regard to symptoms. Samples from western New York (Chautauqua, Erie, and Ontario Counties) consisted of Concord vines collected from 16 different commercial production sites. Additional samples were obtained from the United States Department of Agriculture (USDA) National Plant Germplasm System, Plant Genetic Resources Unit in Geneva, NY. In order to determine the occurrence of GVE in California, samples were obtained from the USDA National Clonal Germplasm Repository in Winters, CA; the Davis Grapevine Virus Collection; the Foundation Plant Services collection; and commercial vineyards in Napa Valley, CA.

Virus detection. Two parallel surveys were undertaken, with the detection approaches taken in New York and California differing. To detect the presence of virus in cane samples from New York, a multiplex macroarray method was employed, as described (Thompson et al. 2014). Additionally, samples were specifically tested for GLRaV-3 by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Bioreba), and for GVE by reverse-transcription polymerase chain reaction (RT-PCR) using primers GVE-1-For and GVE-Rev for the amplification of a 992-bp fragment spanning open reading frame (ORF) 4 and 5 in the genome of the SA94 isolate (GenBank accession GU903012) (Coetzee et al. 2010b). For California samples, nucleic acid (NA) extracts were prepared from each of the grapevine samples as described by Al Rwahnih et al. (2014). About 0.2 g of frozen leaf petioles was homogenized using a HOMEX grinder (Bioreba) and

NA extracts were prepared using a MagMAX-96 viral RNA isolation kit (Invitrogen) per the manufacturer's protocol. Extracted NA samples were analyzed for the presence of GVE by RT-PCR as described above. Samples were also tested for mixed infection with grapevine leafroll-associated viruses and vitiviruses by quantitative RT-PCR using TaqMan probes on the ABI 7900 HT Fast real-time PCR system (Invitrogen), as described previously (Klaassen et al. 2011).

Sequence analysis. To evaluate nucleotide diversity and look for evidence of selection pressures driving the evolution of GVE populations, a 675-nucleotide (nt) RT-PCR region from each isolate was sequenced using primers GVE-1-For and GVE-Rev (Coetzee et al. 2010b). To sequence isolates from New York, the PCR fragment was produced using AccuPrime Taq DNA Polymerase (Life Technologies) and directly sequenced using the amplification primers. For each sample, PCR products from two independent PCR experiments were sequenced and there were no discrepancies observed between sequences. For isolates from California, the amplified PCR products were analyzed by electrophoresis using a 1% agarose gel with Tris-acetate-EDTA buffer. Amplicons of GVE were eluted from gels using the ZymoClean Gel DNA Recovery Kit (Zymo Research Corp), quantified, and sequenced using GVE-1-For and GVE-Rev primers by Sanger sequencing at the University of California-Davis sequencing facility (http://dnaseq.ucdavis.edu).

A multiple alignment was produced using the Muscle algorithm (Edgar 2004). A maximum likelihood tree as implemented in Topali v2 (Milne et al. 2009) was constructed to determine the relationship between the isolates sequenced in this study, as well as their relationships to previously reported North American, African, and Asian isolates. Alternatively, an alignment of a shorter 471-nt region from the previous alignment was designed to include only the coat protein (CP) ORF; this facilitated an alignment with the corresponding sequences from more divergent vitivirus species in order to assess the relative position of GVE in this group. Nucleotide sequence diversity was measured, as defined by Nei (Nei and Li 1979), and evidence of selection pressure was evaluated using a Tajima's D (Tajima 1989); both analyses were conducted as implemented in DnaSP (Librado and Rozas 2009).

Results

A limited survey of viruses in Concord vines in western New York. In order to assess the occurrence of GVE and GLRaV-3 in Concord vines in New York, a limited survey was performed, including samples from production, nursery, and repository vineyards. Of the 46 Concord vines (*V. labrusca*) tested, 10 (22%) showed positive signals for hybridization to oligonucleotide probes for GVE in the macroarray assay (Fig. 1; Table 2). Nucleic acid extracts from vines testing positive for GVE hybridized with from 8 to 20 of the 24 GVE-specific oligonucleotide probes on the array. The presence of GVE sequences in infected plants was confirmed by RT-PCR followed by direct sequencing of the amplicons. Two additional clones of *Vitis* spp. from the USDA germplasm repository in Geneva, NY also tested positive for GVE; these were the *Vitis* hybrid 'Remaily 66-54-2' clone PI588332 and the *V. riparia* clone PI588344 (Table 2). All of the GVE-infected vines in New York also showed positive signals for hybridization to 6 to 25 of the 44 oligonucleotide probes for GLRaV-3. Thus, all GVE-infected Concord vines were coinfected with GLRaV-3, consistent with results from ELISA testing. No other grapevine viruses were detected in this sample of 46 Concord vines.

GVE present in *V. vinifera* cultivars in California. In parallel with the work in New York, 417 California vines (*V. vinifera*) were screened for the presence of GVE and other viruses by RT-PCR. GVE was detected in eight of the clones (approximately 2%), all from germplasm and virus collections (Table 2). None of the 102 vines from commercial vineyards tested positive for GVE. All of the GVE-positive vines also harbored between three to six additional viruses, including at least one leafroll virus and one additional vitivirus.

Sequence analysis. To confirm the presence of GVE and assess the sequence diversity among isolates of this virus, a GVE-specific cDNA was amplified by RT-PCR and sequenced directly or after cloning into pGEM-T (Promega Corp.), and a 675-nt region was analyzed. The analyzed cDNA spanned 471 nt of the 3′ end of the CP gene (ORF4), a 17-nt intergenic region, and 187 nt of the 5′ end of the putative nucleic acid binding protein gene (ORF5) in the genome of GVE-SA94 (nucleotide positions 6,624 to 7,299 in GenBank accession GU903012). All 20 isolates from this study and two described previously (Thompson et al. 2014) were sequenced (GenBank accessions KR062097 to KR062118) and shown to be closely related (>98% nucleotide identity and 100% amino acid identity) to GVE-SA94 from South Africa and to form a separate clade from the Asian isolates TvAQ7 and GFMG-1 (GenBank accessions AB432910 and KF588015, respectively; Fig. 2). This study further supports the relationships between vitiviruses (Alabi et al. 2013), showing GVE to be the most distantly related member of the group (Fig. 2A). Additional analyses revealed very low overall nucleotide diversity ($\pi = 0.005$), and a nonsignificant trend toward negative selection ($D = -1.59$, $0.10 > P > 0.05$).

Discussion

GVE is established in the United States. GVE infection was detected in multiple vines from New York and California; together with a recent report of this virus from Washington State (Alabi et al. 2013), this indicates that the virus is established in the major grape-growing regions of the United States. GVE was observed commonly in

Table 1. Origin of grapevine samples tested for viruses in this study

Collection sites, regions[a]	Species	Number of vines
Western New York	*Vitis labrusca* (Concord)	44
USDA NPGS, Plant Genetic Resources Unit, Geneva, NY	*V. riparia*	1
	V. hybrid	1
	V. labrusca (Concord)	2
University of California, Davis Grapevine Virus Collection	*V. vinifera*	198
USDA NPGS, National Clonal Germplasm Repository, Davis, CA	*V. vinifera*	77
Foundation Plant Services, Davis, CA	*V. vinifera*	40
Napa Valley, CA	*V. vinifera*	102

[a] USDA NPGS = United States Department of Agriculture National Plant Germplasm System.

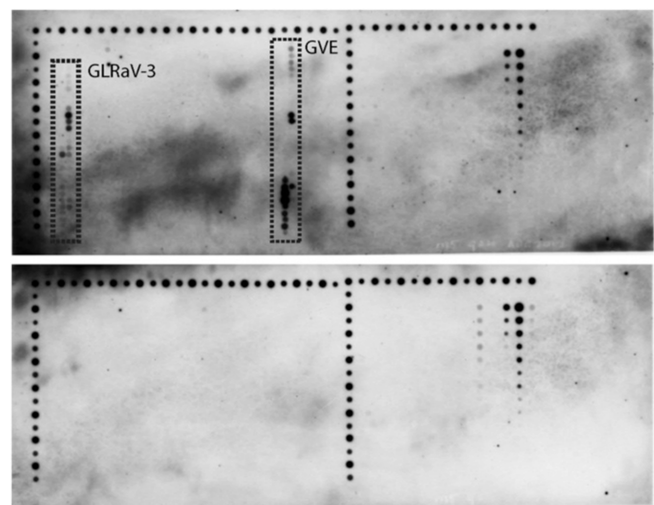

Fig. 1. Macroarray detection of grapevine viruses. Above, a nucleic acid extract from *Vitis labrusca* 'Concord' sample MacsR97 was purified, labeled, hybridized to the nylon membrane, and resolved as described by Thompson et al. (2014). Boxed areas show sections of the membrane with probes specific to *Grapevine leafroll-associated virus-3* (GLRaV-3) and *Grapevine virus E* (GVE) as labeled). Hybridization signals along the top and in vertical columns are those of the macroarray controls. Below, a macroarray of a negative control nucleic acid extract from the reference Concord vine DC1-1, processed as described above.

Concord vines in New York, being found in 22% of the 46 vines tested. The detection of infected vines in commercial vineyards in New York highlights the potential for spread, because these vines represent reservoirs from which the virus could be transmitted to other production vineyards (secondary spread). Although GVE was detected in California, it was seen only in grapevine collections, not in commercial vines. The limited scope of this survey precludes an assessment of incidence or distribution.

Table 2. Mixed infection of *Grapevine virus E* and other viruses in infected grapevines from New York and California

Samples	Host	Source[a]	Viruses detected[b]
New York			
DAA number 1	*Vitis labrusca*	Commercial vineyard	GLRaV-3, GVE
DAA number 4	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
DAA number 5	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
DAA number 6	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
HAA number 9	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
DNSP	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
VD1-1	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
MacsR97	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
BW3-1	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
BH1-3	*V. labrusca*	Commercial vineyard	GLRaV-3, GVE
10-102	*V. riparia*	NPGS, Geneva	GLRaV-3, GVE
5-2	*V.* hybrid	NPGS, Geneva	GLRaV-3, GVE
California			
ARM VSV 11 V25	*V. vinifera*	DGVC	GLRaV-3, GRSPaV, GVA, GVE
ARM VSV 12 V22	*V. vinifera*	DGVC	GLRaV-1, -2, -3, GRSPaV, GVA, GVE
K5-56	*V. vinifera*	NPGS, Davis	GLRaV-1, -2, -3, -5, GRSPaV, GVA, GVE
K7-57	*V. vinifera*	NPGS, Davis	GLRaV-2, -3, GRSPaV, GVA, GVD, GVE
K8-45	*V. vinifera*	NPGS, Davis	GLRaV-2, -3, GVA, GVB, GVD, GVE,
K8-46	*V. vinifera*	FPS, Davis	GLRaV-2, -3, GVA, GVB, GVD, GVE
K8-53	*V. vinifera*	NPGS, Davis	GLRaV-2, -3, GVA, GVB, GVD, GVE
K8-59	*V. vinifera*	NPGS, Davis	GLRaV-1, -3, GVA, GVB, GVD, GVE

[a] NPGS = National Plant Germplasm System, DGVC = Davis Grapevine Virus Collection, and FPS = Foundation Plant Services.
[b] GLRaV-1, -2, and -3 = *Grapevine leafroll-associated virus-1, -2,* and *-3*; GRSPaV = *Grapevine rupestris stem pitting-associated virus*; GVA = *Grapevine virus A*; GVB = *Grapevine virus B*; GVD = *Grapevine virus D*; and GVE = *Grapevine virus E*.

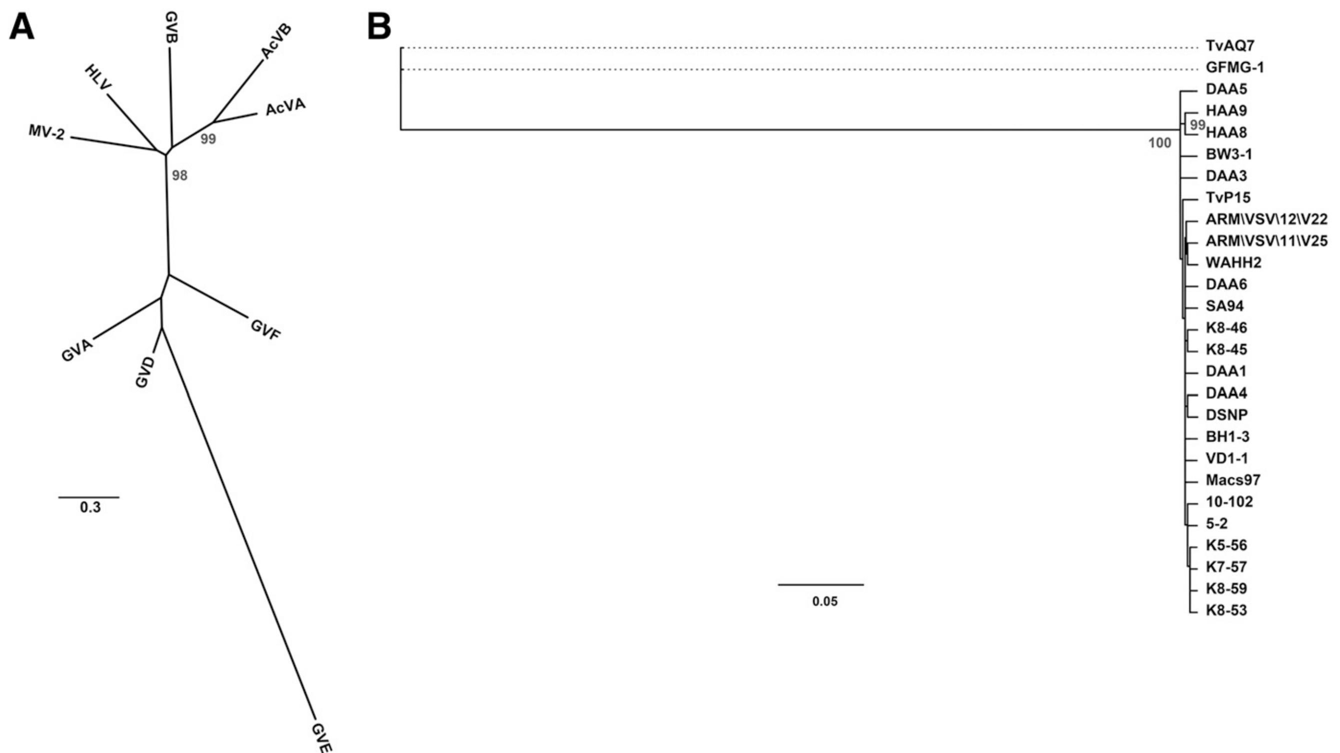

Fig. 2. A, Unrooted maximum likelihood tree (model K80+G) (Kimura 1980) of a fragment of the coat protein open reading frame showing the evolutionary relationships of vitiviruses. Bootstrap values correspond to 100 repetitions. Only values above 70 are shown. Virus acronyms (and corresponding accession number) are AcVA, *Actinidia virus A* (JN427014); AcVB, *Actinidia virus B* (NC_016404); GVA, *Grapevine virus A* (NC_003604); GVB, *Grapevine virus B* (NC_003602); GVD, *Grapevine virus D* (JQ031715); GVE (GU903012); GVF, *Grapevine virus F* (NC_018458), HLV, *Heracleum latent virus* (×79270); and MV-2, *Mint virus 2* (AY913795). **B,** Unrooted maximum likelihood tree (model TVMef+G (Posada 2003)) of a 675-nucleotide fragment of the GVE genome showing the evolutionary relationships between GVE isolates from North America, South Africa, and Asia. Bootstrap values correspond to 100 repetitions. Only values above 70 are shown. Isolate acronyms and corresponding accession numbers are GVE-GFMG-1 (KF588015), GVE-TvAQ7 (AB432910), TvP15 (AB432911), GVE-WAHH2 (JX402759), and GVE-SA94 (GU903012).

GVE host range and effects. GVE has previously been reported as infecting cultivars of *V. labrusca* (Nakaune et al. 2008; Thompson et al. 2014) and *V. vinifera* (Alabi et al. 2013; Coetzee et al. 2010a). In this study, GVE was detected in cultivars of these two species, and additionally from *V. riparia* and a *Vitis* hybrid. For GVE (and other vitiviruses), there are no reported hosts or reservoirs outside of *Vitis* spp. although, under experimental conditions, some vitiviruses can be mechanically transmitted to and propagated in herbaceous hosts (du Preez et al. 2011; Nakaune et al. 2008). GVE was shown to be transmitted by the mealybug *Pseudococcus comstocki* in Japan (Nakaune et al. 2008). Thus, GVE has persisted in infected vines and has the potential to spread in planting stocks but, at present, there is no indication of spread by mealybugs in North America. Vitiviruses, in particular GVA and GVB, are associated with rugose wood disease symptoms (Goszczynski and Jooste 2002; Habili and Randles 2012; Martelli and Boudon-Padieu 2006; Rosa et al. 2011), although inferring causal relationships through a demonstration of Koch's postulates is lacking for these and most other viruses in grapevine. The extent to which GVE affects vine health and productivity alone or in combination with other viruses remains to be determined.

Genetic diversity of GVE in the United States is limited. The GVE isolates described in this study exhibit limited genetic diversity. A comparison of the GVE sequences from this study with those available in public databases showed U.S. isolates to be very closely related to the SA94 isolate from South Africa (Coetzee et al. 2010b). Worldwide, there are two distinct genetic lineages of GVE among the fully sequenced genomes. One lineage is represented by isolates SA94 from South Africa (GenBank accession GU903012) and WAHH2 from the United States (GenBank accession JX402759). The second lineage is typified by the isolates TvAQ7 from Japan (GenBank accession AB432910) and GFMG-1 from China (GenBank accession KF588015). The nucleotide sequence identity within groups is >98% (100% CP amino acid identity), while identities between groups is only 70% (87% CP amino acid identity). Results from this study suggest that only the SA94/WAHH2 genetic lineage is present in the United States, because no isolates similar to the TvAQ7 and GFMG-1 isolates from Japan and China, respectively, were detected. Interestingly, with the recent submissions of sequences of new GVE isolates from China (GenBank accessions KF588017 to KF588034), it is apparent that both genetic lineages are present in this region. The limited sequence identity between lineages of GVE highlights the necessity of using robust diagnostic primers to avoid false negatives in germplasm testing.

The methodologies used to detect GVE in New York and California differed and each have their limitations. The macroarray method is less sensitive than PCR but is relatively robust in detecting virus strain sequence variants (Agindotan and Perry 2007; Thompson et al. 2014). For the GVE isolates detected, hybridization signals were very strong and sensitivity was not limiting. PCR is more sensitive but is sequence specific and may fail to detect sequence variants. After the conclusion of this work, it was brought to our attention that the primer GVE-1-For designed by Coetzee et al. (2010b) has a 3′-terminal mismatch to the second lineage isolates described in the literature (e.g., isolates TvAQ7 and GFMG-1). This might have resulted in a failure to detect some isolates in California but not isolates in New York, where the primary detection method was the macroarray.

All GVE-infected vines are coinfected with other viruses. Concord vines in New York infected with multiple vitiviruses were reported by Thompson et al. (2014). In the present survey, using the same methodology, GVE was the only vitivirus observed in Concord vines, and all GVE-positive Concord samples were also infected with GLRaV-3. An early coinfection of planting stocks commonly used among growers and nursery operations in New York could explain this observation. In a Concord vine survey conducted in Washington state, GVE was not tested for and no vitiviruses were detected but GLRaV-3 was the most prevalent leafroll-associated virus observed (Bahder et al. 2013), consistent with the results from New York. By contrast, in *V. vinifera* samples from California, infections with multiple vitiviruses were observed, and all GVE-positive samples were coinfected with at least one other vitivirus and a leafroll-associated virus (Table 2). Virus cotransmission among grapevines carried out by mealybug vectors has been demonstrated with vitiviruses and ampeloviruses (Hommay et al. 2008; Le Maguet et al. 2012) and this would result in multiple virus infections, as observed in the *V. vinifera* surveyed in this study.

This study demonstrates that GVE is established in commercial plantings of Concord vines in the eastern United States. Whether GVE is also present in Concord vines in the western United States remains to be determined. This virus was also observed in field-planted collections of *V. vinifera* in California but it has not been reported from commercial vineyards. Detection methods based on RT-PCR are currently used to screen for GVE in foundation stocks, and these efforts will help to limit the additional spread of this virus.

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