Description of a Novel Monopartite Geminivirus and Its Defective Subviral Genome in Grapevine

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ABSTRACT

A novel virus was detected in grapevines by Illumina sequencing during the screening of two table grape (*Vitis vinifera*) accessions, cultivars Black Beet and Nagano Purple, from South Korea. The monopartite circular ssDNA genome sequence was subsequently confirmed by rolling cycle amplification, cloning and Sanger sequencing. The complete viral genomic sequence from both accessions ranged from 2,903 to 2,907 nucleotides in length and contained the conserved nonanucleotide sequence TAATATT↓AC and other sequence features typical of the family *Geminiviridae*, including two predicted sense and four complementary-sense open reading frames. Phylogenetic analysis placed the novel virus in a unique taxon within the

family *Geminiviridae*. A naturally occurring defective subviral DNA was also discovered. This defective DNA molecule carried a deletion of approximately 46% of the full-length genome. Both the genomic and defective DNA molecules were graft-transmissible although no disease is yet correlated with their occurrence in *Vitis* spp. The tentative names Grapevine geminivirus A (GGVA) and GGVA defective DNA (GGVA D-DNA) are proposed. PCR assays developed using primers designed in the coat protein gene led to the detection of GGVA in 1.74% of 1,262 vines derived from 15 grapevine cultivars from six countries across three continents.

Grapes (*Vitis* spp.) are one of the world's most ancient fruit crops. They are sold fresh as table grapes or used to produce wine, raisins, jelly, juice, and other grape products thus generating significant economic activity worldwide. In the United States, California is by far the leading producer of grapes and grape products, accounting for 90% of the bottled wines (<http://www.wineinstitute.org>) and almost 100% of table grapes and raisins produced in the country (<http://www.grapesfromcalifornia.com/>). Due to the continued recognition of the threat of viruses to the sustainability of the grapevine industry, considerable investments are being made to improve the capacity to accurately and rapidly identify and characterize potentially economically damaging viruses. Additionally, efforts are made to control their impact before they become widespread and high-throughput sequencing (HTS) technology has the potential to enhance these control efforts. The comprehensive nature of HTS analysis, its analytical speed, and improved sensitivity over traditional methods improve surveys of recognized viruses and the discovery of novel viruses (Al Rwahnih et al. 2015).

With over 70 infectious agents identified and characterized across the globe, grapevine has the distinction of being host to the highest number of intracellular pathogens of any crop (Martelli 2014). Most of these infectious agents are viruses with ribonucleic acid (RNA) genomes (Maliogka et al. 2015a; Martelli 2014). However, three viruses with deoxyribonucleic acid (DNA) genomes have also been documented from grapevines within the last decade. This further complicates the already complex virome in the vineyard. The grapevine-infecting DNA viruses are *Grapevine vein clearing virus* (GVCV; genus *Badnavirus*; family *Caulimoviridae*)

(Zhang et al. 2011), *Grapevine red blotch-associated virus* (GRBaV; family *Geminiviridae*) (Al Rwahnih et al. 2012a; Krenz et al. 2012; Poojari et al. 2013), and grapevine Roditis leaf discoloration-associated virus (GRLDaV; genus *Badnavirus*) (Maliogka et al. 2015b). GRBaV has been shown to be widespread in multiple cultivars across several grapevine-growing states of the United States (Al Rwahnih et al. 2013; Krenz et al. 2014) and is linked to reduced productivity of affected vines (Sudarshana et al. 2015) akin to the negative impacts reported for grapevine leafroll disease (Alabi et al. 2016a). Due to the recognition of the increasing importance of members of the family *Geminiviridae* as serious threats to global agriculture (Briddon 2015), the discovery of GRBaV has generated tremendous interest in DNA viruses within the global community of grapevine virologists.

The viruses belonging to the family *Geminiviridae* are characterized by their circular, single-stranded DNA (ssDNA) genomes encapsidated in geminate (twinned) incomplete icosahedral particles (Brown et al. 2012). Monopartite geminiviruses possess a single copy of the ssDNA genome (resembling the DNA-A component of begomoviruses) that typically encodes two virion-sense (V1 and V2) and two to four complementary-sense (C1 to C4) genes. Geminiviruses with bipartite genomes possess two ssDNA genome components (DNA-A and DNA-B components). The DNA-B component encodes one gene each in the virion- (BV1) and complementary-sense (BC1) orientations. Although some of the most recently discovered geminivirus-like sequences, particularly those characterized from perennial host plants, have relatively bigger genomes, each genome component ranges in size from 2.5 to 3.0 kb (Brown et al. 2012). To date, seven genera that differ considerably in their genome organization and biological properties have been recognized in the family *Geminiviridae* (Varsani et al. 2014). A unique commonality across species within the different genera in the family is the presence of a conserved nonanucleotide sequence motif "TAATATT↓AC" at the origin of virion-strand replication within the intergenic region (Brown et al. 2012). The only exceptions are members of the genus *Becurtovirus* and *Eragrostis curvula streak virus* which have two nucleotide

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary figures are published online.

substitutions at positions four (T to G) and eight (A to C) of their nonanucleotide sequence motifs.

In addition to their monopartite or bipartite ssDNA genome components, some geminiviruses may have additional subgenomic defective DNA (Stenger et al. 1992) and/or satellite DNA (Briddon et al. 2003; Saunders and Stanley 1999; Zhou 2013). These are ssDNA molecules that are typically half the size of the genomic component and may be associated with symptom modulation in the host plant (Patil and Dasgupta 2006). The defective molecules may be derived from either of the two genomic components or even from the satellite DNA molecule. Typically, the defective molecules derived from the DNA-A component contain the intergenic region with all the cis-acting sequences required for replication (including the “TAATATT↓AC” nonanucleotide sequence motif), the N-terminal sequences of the Rep-associated protein and sometimes a small part of gene encoding coat protein (CP) (Patil and Dasgupta 2006). Those derived from the DNA-B component, on the other hand, typically contain part of the BC1 protein but lack the BV1 gene (Patil and Dasgupta 2006). Studies have shown that the defective molecules can be encapsidated by the geminivirus CP to form isometric particles (Bottcher et al. 2004; Casado et al. 2004; Frischmuth et al. 2001) thus enabling their transmission by the insect vector (Czosnek et al. 1989). Defective nucleic acid (RNA or DNA) molecules may or may not interfere with the replication of their parental (helper) virus genomic component (Patil and Dasgupta 2006; Simon et al. 2004). Those that impair the replication of the helper virus are referred to as defective interfering (DI-DNA) molecules while those that do not (or whose biological functions are yet to be determined) are simply called defective (D-DNA) molecules. To date, no D-DNA or DI-DNA molecule has been characterized from grapevines to the best of our knowledge.

In the present study, we report the natural occurrence and discovery of a novel ssDNA virus, tentatively named Grapevine geminivirus A (GGVA), and of a putative defective DNA molecule (GGVA D-DNA) associated with the virus in one of two isolates. This is the first evidence for the natural occurrence of a D-DNA molecule in grapevines. Both the viral genomic and defective DNA molecules are graft, but not mechanically, transmissible.

MATERIALS AND METHODS

Plant material. In 2013, two accessions of table grapes (*Vitis vinifera*), cultivars Black Beet (BB) and Nagano Purple (NP), were received as dormant cuttings from South Korea under a USDA-APHIS-PPQ588 controlled import permit for inclusion in the Foundation Plant Services (FPS, UC-Davis, CA) Public Collection. Representative canes of each accession were propagated on their own root as two node cuttings under mist and maintained in FPS quarantine growing facilities. Four plants of each cultivar were maintained within an insect-proof greenhouse enclosure. The plants were observed for virus-like symptoms for 2 to 3 months after bud break and leaf tissue (with petioles intact) was sampled for virus detection.

Assays for known grapevine pathogens. Total nucleic acid (TNA) extracts were prepared from the NP and BB source vines as described by Al Rwahnih et al. (2012b). Briefly, approximately 0.2 g of leaf petioles was homogenized using a HOMEX grinder (ThermoFisher Scientific, Grand Island, NY) and TNA extracts were prepared using a MagMAX-96 viral RNA isolation kit (ThermoFisher Scientific) as per manufacturer's protocol. Extracted TNA samples were analyzed by reverse transcription quantitative PCR (RT-qPCR) using TaqMan probes on the QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific) as described previously (Osman et al. 2008; Klaassen et al. 2011). The samples were screened for the following pathogens: *Grapevine leafroll-associated virus-1* (GLRaV-1), *Grapevine leafroll-associated virus-3* (GLRaV-3), and *Grapevine leafroll-associated virus-4*

(GLRaV-4, plus strains 9 and Car) (genus *Ampelovirus*); *Grapevine leafroll-associated virus-2* (GLRaV-2, plus strain 2RG; genus *Closterovirus*); *Grapevine leafroll-associated virus-7* (GLRaV-7; genus *Velarivirus*); *Grapevine fleck virus* (GFKV; genus *Maculavirus*); *Grapevine rupestris vein feathering virus* (GRVfV; genus *Marafivirus*); *Grapevine fanleaf virus* (GFLV), *Tobacco ringspot virus* (TRSV), and *Tomato ringspot virus* (ToRSV) (genus *Nepovirus*); *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD), and *Grapevine virus E* (GVE) (genus *Vitivirus*); *Grapevine rupestris stem-pitting associated virus* (GRSPaV; genus *Foveavirus*); phytoplasmas and *Xylella fastidiosa*, the causative agent of Pierce's disease. The vines were also screened for the recently described Grapevine fabavirus (GFabV) (Al Rwahnih et al. 2016).

Biological assays. Since symptoms atypical of currently known grapevine viruses were observed only on vines of cultivar NP (Fig. 1A), assays were conducted to determine if the putative etiological agent(s) associated with the observed symptoms is graft- and/or mechanically transmissible. Bud chips from symptomatic NP vines and asymptomatic BB vines were grafted onto Cabernet franc (*V. vinifera*) test plants as described in Al Rwahnih et al. (2015). In addition, leaf petiole tissue from NP and BB vines was mechanically inoculated onto a panel of herbaceous indicator plants including *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Cucumis sativus*, and *Nicotiana clevelandii* as previously described (Rowhani et al. 2005).

HTS and sequence analysis. Aliquots of TNA samples from the NP and BB source plants were subjected to ribosomal RNA (rRNA) depletion and cDNA library construction using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina). Sequencing was performed on the Illumina NextSeq 500 platform at the SeqMatic LLC sequencing facility (Fremont, CA) and the raw HTS reads were trimmed using the CLC Bio Genomic Workstation (Qiagen, Hilden, Germany) trimming tool. Contiguous consensus sequences “contigs” were then assembled from the non-*Vitis* reads using the CLC assembler (200 bp minimum). The NCBI blastx program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to compare contigs to reference virus genomes using parameters that were chosen to match the default parameters of the CLC Bio Genomics Workstation including a mismatch cost of 2, indel costs of 3, and a similarity fraction of 0.8 over a length fraction of 0.5. An E-value cutoff of 1e-3 was used to reduce the chance of false positives.

Rolling cycle amplification (RCA), PCR validation, and full-length amplification of viral genomic DNA molecule.

To confirm the HTS result, TNA aliquots from the NP and BB source vines were subjected to RCA (Inoue-Nagata et al. 2004) using a TempliPhi Amplification Kit (GE Healthcare Life Sciences, Uppsala, Sweden) as described in the manufacturer's protocol. The RCA products were monomerized with *SbfI* and *HincII* restriction enzymes (New England BioLabs, USA) that were predicted to be single cutters and selected based on in silico analysis of the derived HTS sequences performed with the NEBcutter V2.0 software (<http://nc2.neb.com/NEBcutter2/>). The digested products were separated by electrophoresis on a 1.5% agarose gel and Tris-acetate-EDTA (TAE) buffer and DNA bands in the approximately 3 kb range were gel-eluted with the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA) from each sample. The full viral genome sequence was determined in two independent labs using two complementary approaches. In the first approach conducted at the FPS facility, UC-Davis, CA, the full-length viral genome was amplified with Phusion DNA polymerase (ThermoFisher Scientific) using a TNA template and a pair of abutting primers GGVAv950/GGVAc961 (Table 1). The ~3-kb viral genomic DNA fragments obtained from NP and BB vines were independently gel-eluted as described above and directly subjected to Sanger sequencing using the same primers at the UC-Davis sequencing facility (<http://dnaseq.ucdavis.edu>). Based on the

initially obtained sequences, additional primers (data not shown) were designed and utilized for genome-walking using the same TNA template for each sample. In the second approach conducted at the Texas A&M AgriLife Research & Extension Center facility, another pair of abutting primers GGVAv1402/GGVAc1438 was designed from the assembled HTS sequences and utilized in PCR to

amplify the complete viral genomic DNA molecule from NP and BB using the PrimeSTAR GXL DNA polymerase (Clontech Laboratories, Inc.) and the RCA eluates as template. The obtained DNA amplicons were cloned individually into the pCR2.1 TOPO-TA vector and transformed into One Shot TOP10 chemically competent *Escherichia coli* cells according to the manufacturer's

Fig. 1. Symptoms observed on source vines of *Vitis vinifera* **A**, ‘Nagano Purple’ (NP) and **B**, ‘Black Beet’ (BB) and corresponding vines of Cabernet franc (Cf) graft-inoculated with bud chips from NP (**C**) and BB (**D**) vines. The chlorotic ringspot symptoms observed on the source NP vine (**A**) was reproduced on the graft-inoculated Cf vine in mixture with leafroll symptoms (**C**) whereas only leafroll symptoms were produced on Cf vine (**D**) graft-inoculated with material from source BB vine (**D**).

TABLE 1. Primers and probes used in the study for the characterization and routine diagnosis of Grapevine geminivirus A (GGVA) and its defective DNA molecule (GGVA D-DNA)

Primer name	Target molecule	ORF	Sequence (5'-3') ^a	T (°C)	Amplicon size (bp)	Reference
GGVAv541	Genomic DNA and D-DNA	V1	ggCCAAGAgAAggTTgACCTAT	56	384	This study
GGVAc924		V1	CATgTggggTTATgCTgATC			
GGVAv2097		C1	CgTCCTTgAATCTCCCTCTA			
GGVAc239	Genomic DNA and D-DNA	IR	gTgggACCACATAACCgACA	60	63	This study
GGVAv412		V2	AAgggAACgGCCAACA			
GGVAc474		V1	gATTTCCTCTTCTCggATTgAAg			
Probev430	Parental virus and D-DNA	V1	CTCATAgATTggATCCATgg	56	2,905	This study
GGVAv950		V1	AAgTCCTACCACgTgCTgAg			
GGVAc961		V1	TggTAaggACTTgTCATCgA			
GGVAv1402	Genomic DNA alone	C3	CgTCCAgACCCTgTATgTTATC	55	2,905	This study
GGVAc1438		C3	gCTACACAAATgCTggATAAC			
GGVA D-DNAv1246		C1	CAC TTCgAAACTTgAAATCCC			
GGVA D-DNAc1279	D-DNA alone	C1	gAgATTCAAggACgggATT	55	1,559	This study
PBL1v2040		BC1	gCCTCTgCAGCARTgRTCKATCTTCATACA			
PCRc1		IR	CTAgCTgCAGCATATTTACRARWATgCCA			
PCRc154	DNA-β	IR	ggTAATATTATAHCggATgg	50	600	Rojas et al. 1993
Beta01		IR	ggTACCACTACgCTACgCAGCAGCC	50	750	
Beta02		IR	CACATggggACCCTCCCATCCATgg	50	1,350	

^a The following IUB group codes were used to identify redundancies: K = G + T, W = A + T, R = A + G, H = A + C + T.

protocols (Life Technologies). Screening, selection, plasmid isolations and sequencing of positive transformants were performed as described previously (Mulenga et al. 2016). An additional pair of primers (data not shown) was designed to genome-walk the plasmid DNA samples to obtain the complete viral DNA genome sequences from each isolate. Using this approach, the complete virus genome was also determined from three additional grapevine cultivars.

Search for the presence of additional genomic components and subviral agents. The occurrence of genomic components possibly corresponding to DNA-B and DNA- β satellite DNA molecules was investigated. For this purpose, two pairs of degenerate DNA-B-specific primers (Rojas et al. 1993) and a pair of DNA- β -specific primers (Briddon et al. 2002) were used in PCR. Additionally, an ~ 1.5 -kb DNA band representing a unique restriction digestion product produced by *HincII* digestion only from isolate NP (Fig. 2A) was gel-eluted, A-tailed, cloned, and sequenced as described previously (Alabi et al. 2016b). Based on the sequences obtained, a pair of abutting primers GGVA D-DNAv1246/GGVA D-DNAc1279 was designed from aligned sequences of the subviral DNA molecule and the full-length viral DNA sequences (obtained above) to specifically target the subviral DNA molecule. The amplified ~ 1.5 -kb DNA band (Fig. 2B) was cloned and sequenced as described above.

Analysis of viral and subviral DNA sequences. Sequence fragments corresponding to each DNA amplicon were edited and assembled as described previously (Alabi et al. 2016b). Each assembled DNA molecule was subjected to blastn analysis (Altschul et al. 1990) to confirm its viral origin and to search the global database for homologous sequences. The programs ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) and Pfam (<http://pfam.xfam.org/>) were used for the identification of conserved protein domains (Finn et al. 2014) encoded by each genomic DNA molecule. The sequence matches for each predicted protein product were determined using the SmartBLAST algorithm of the ORF Finder. Nucleotide and amino acid sequence alignments were generated with the MUSCLE alignment program (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and the aligned sequences were used for pairwise comparisons and phylogenetic analysis using the approaches and suite of programs described in Alabi et al. (2016b).

Phylogenetic analyses were conducted using the neighbor-joining, minimum evolution, and maximum likelihood methods from Molecular Evolutionary Genetic Analysis software MEGA

version 6 (Tamura et al. 2013) based on complete genome nucleotide sequences and derived coat protein and replication-associated protein amino acid sequences of the viral genomic DNA. Only the neighbor-joining trees are presented since all three methods generated similar tree topologies.

The software SnapGene Viewer version 3.1.4 (<http://www.snapgene.com/>) was used to draw the schematic representation of the circular genomes of the genomic and subviral DNA molecules and the NucPred program (Brameier et al. 2007) was used to identify the CP-encoded nuclear localization signal of the novel virus.

Development of conventional and Taqman qPCR assays for detection of GGVA.

To enable routine diagnosis of the novel virus in epidemiological studies and to explore its prevalence, complete virus genome sequences of seven GGVA isolates (GenBank accession numbers KX570607 to KX570618) aligned as described above were used to design a primer pair from the coat protein (CP) gene (V1 ORF) for conventional PCR and Taqman-based qPCR assays (Table 1). The primer pair GGVAv541/GGVAc924 was designed to amplify 384-bp CP-specific DNA fragments from the viral genomic and defective DNA molecules by conventional PCR (Table 1). For the qPCR assay, the primer pair GGVAv412/GGVAc474 along with the probe GGVAv430 was designed to drive the amplification of a 63-bp DNA fragment specific to the CP gene of GGVA (Table 1). The design of primers and probe for the qPCR assay was carried out under criteria set by the Primer Express software (ThermoFisher Scientific) and following the minimal criteria for the design of qPCR assays (Bustin et al. 2009). All qPCR probes were MGB probes, labeled with 5' FAM reporters and a nonfluorescent quencher. Nucleic acid extracts were prepared from leaf petioles as described above and amplification conditions for the conventional PCR assay consisted of one cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min; and one cycle of 72°C for 5 min. DNA amplicons obtained in conventional PCR were analyzed by agarose gel electrophoresis in TAE buffer and the virus-specific sequences of a select number of amplicons were verified via direct sequencing of gel-eluted DNA fragments at the UC-Davis sequencing facility (<http://dnaseq.ucdavis.edu>). Cycling conditions for qPCR were 50°C for 2 min, 95°C for 20 s, the 40 cycles at 95°C for 1 s and 60° for 20 s. All samples were analyzed on a QuantStudio 6 Flex Real-Time PCR System

Fig. 2. A, Restriction enzyme digestion of rolling cycle amplification products of DNA extract from *Vitis vinifera* 'Nagano Purple' (NP) and 'Black Beet' (BB). Both enzymes *SbfI* and *HincII* produced a DNA product in the ~ 3 -kb size range from NP and BB, whereas the enzyme *HincII* produced additional bands of ~ 1.5 -kb only from NP. **B,** PCR amplification of virus-specific bands corresponding to the Grapevine geminivirus A genomic (GGVA) and defective (GGVA D-DNA) molecules from NP and BB vines using pairs of molecule-specific abutting primers (Table 1). A third pair of abutting primers could simultaneously amplify both GGVA and GGVA D-DNA when present in the same NP vines. M: 1-kb Plus DNA Ladder (ThermoFisher Scientific).

(ThermoFisher Scientific). The data were analyzed both quantitatively by measuring the quantitative cycle (Cq) in a Microsoft Excel program and graphically by an amplification plot. The Cq is defined as the cycle in which there is a significant increase in reporter signal above the threshold; therefore, it is related to the initial amount of DNA target in the sample (Bustin et al. 2009).

Standard curves were generated by creating five sets of 10-fold serial dilutions and plotting the Cq value against the logarithm of the concentration for the exponential phase of the reaction. A trend line was added to this data by simple linear regression (Bustin et al. 2009). The amplification efficiency (E) of all assays was calculated from the slope of a standard curve generated on a 10-fold dilution in triplicate for every cDNA sample using the formula $E = 10(-1/\text{slope})$.

Screening for GGVA. A total of 1,262 vines were sampled from the National Clonal Germplasm Repository (NCGR), the Davis Grapevine Virus Collection (DGVC), the FPS Introduction Pipeline, and commercial vineyards. The NCGR sampling was biased in favor of source vines introduced from Asian countries since the initially characterized virus isolates NP and BB originated from this region.

RESULTS

Plant material. Systemic virus-like chlorotic ringspot symptoms were observed on young to mid-age leaves of all four potted NP vines (Fig. 1A). In contrast, no symptoms were observed on leaves of all four potted BB vines of the same age (Fig. 1B). Since the chlorotic ringspot symptoms observed on the NP vines are not characteristic of known grapevine viruses, it was hypothesized that the *V. vinifera* cultivar NP might harbor a new uncharacterized virus, thus making this sample a prime candidate for HTS. Cultivar BB was also advanced for HTS for comparative analysis to further decipher the etiology of the disease observed on the NP vines.

Virus detection. Results of the RT-qPCR screening for known grapevine pathogens and the HTS analysis led to the detection of multiple virus and viroid species in mixed infections of different proportions in NP and BB vines. Four viruses (GLRaV-3, GFkV, GSyV-1, and GFabV) (Al Rwahnih et al. 2016) and two viroid species (*Hop stunt viroid* and *Grapevine yellow speckle viroid-1*) were detected in both NP and BB. In addition, GLRaV-2 was detected in NP while GVE, GRSPaV, and GRVfV were detected in BB vines. Both vines were negative for the eleven other infectious agents (GLRaV-1, GLRaV-4, GLRaV-7, GFLV, TRSV, ToRSV, GVA, GVB, GVD, phytoplasmas, and *X. fastidiosa*) that were included in the pathogen screen assays.

Analysis of HTS data. The total number of trimmed sequence reads recovered from NP and BB cDNA libraries ranged from 32 to 34 million and their analysis resulted in the assembly of 35,030 to 35,770 contigs from each library. The annotation and tblastx analyses of these contigs against the reference virus genomes in the NCBI database led to the recovery of genome sequences of all

known grapevine viruses that were previously detected from both vines by RT-qPCR as stated earlier. Further analysis of the HTS contigs led to the recovery of the near complete RNA1 and RNA2 sequences of the recently described Grapevine fabavirus in both NP and BB vines (Table 2) (Al Rwahnih et al. 2016). The first indication of the occurrence of geminivirus-like contigs in the cDNA libraries derived from isolates NP and BB is the recognition of the highly conserved geminiviral nonanucleotide sequence “TAATATT↓AC” among the non-*Vitis* contigs derived from both isolates. Subsequently, multiple geminivirus-specific contigs were recovered from both NP and BB vines (Table 2). A blastx analysis of the approximately 2,905 nucleotides common to both isolates confirmed its geminiviral origin and produced significant alignments with several replication-associated putative conserved domains (data not shown).

Identification of a novel DNA virus and its defective DNA molecule in grapevine. The HTS results were independently validated via recovery of the complete genome of the novel DNA virus from isolates NP and BB by Sanger Sequencing performed either on direct PCR amplicons (Fig. 2B) or plasmid DNA from cloned DNA products that were obtained with abutting primers (Table 1) as described above. Analysis of multiple independent clones from isolates NP (three clones) and BB (four clones) yielded complete ssDNA genome sequences (GenBank accession numbers KX570607 and KX560609) that ranged in size from 2,903 to 2,907 nt and were almost identical (98 to 100%) to each other and to corresponding HTS-derived sequences. The 2% divergence among the nine NP and BB GGVA clones was mostly a result of variations in the lengths of the intergenic region sequences. The complete viral genomic DNA sequences obtained from nine additional cultivars (GenBank accession numbers KX570610 to KX560618) were of the same size range and identity levels as sequences derived from isolates NP and BB. In addition, cloning and sequence analysis of the ~1.55-kb *HincII*-derived DNA fragment from NP (Fig. 2A), and subsequent independent validation using a pair of abutting primers specifically designed for the subviral DNA molecule, led to the recovery of a naturally occurring defective form of GGVA that is about half the size (1,559 nt) of the full-length viral genomic DNA sequence. This subviral molecule is tentatively named GGVA D-DNA since its role in virus-host modulation is yet to be described. In agreement with the results of the restriction enzyme digestion assays, GGVA D-DNA was detected only in NP (but not BB) by PCR using the pair of abutting primers capable of specific detection of the defective DNA molecule (Table 1). Analysis of the subviral DNA molecule showed that it was derived directly from, and arose as a result of, deletions of ~46% of the genome sequence of the monopartite GGVA (parental) virus (Supplementary Fig. S1). Further analysis of all four independent NP clones of GGVA D-DNA (GenBank accession number KX570608) revealed that they are 100% identical to each other but shared only 52 to 53% genome length identities with the parental virus due to the deletion in the rep-associated sequences. No insertion from any other source was found within the genome sequence of GGVA D-DNA. Several attempts to amplify a DNA-B component and the DNA-β satellite molecule from both isolates using published universal primers and PCR conditions (Rojas et al. 1993; Briddon et al. 2002) were not successful, indicating a lack of association of both molecules with GGVA. These results suggest that GGVA is a monopartite ssDNA virus associated with a defective ssDNA molecule.

Genome organization and molecular properties of GGVA. Like most members of the family *Geminiviridae*, the stem-loop sequence of the intergenic region of GGVA contains the non-anucleotide sequence “TAATATT↓AC” (Fig. 3C). A blastn search of the complete genome sequences of GGVA confirmed its geminiviral origin and the most resemblance to several members of the genus *Begomovirus* albeit at low genome coverage (66 to 82% identity; query coverage, 12 to 38%; E value, 1e-62 to 4e-113). Two

TABLE 2. Recovery of sequence reads representing novel viruses detected from grapevine (*Vitis vinifera*) accessions Nagano Purple (NP) and Black Beet (BB) via high-throughput sequencing

Sample	Virus ^a	Mapped reads	Contigs	
			Number	Size
NP	GGVA	1,568	3	271–1,164
	GFabV RNA1	71,280	2	1,615–4,077
	GFabV RNA2	33,927	2	1,224–1,852
BB	GGVA	2,940	4	266–1,063
	GFabV RNA1	117,165	1	3,082
	GFabV RNA2	157,433	1	5,755

^a GGVA, Grapevine geminivirus A; GFabV, Grapevine fabavirus (Al Rwahnih et al. 2016).

virion-sense (V1 and V2) and four complementary-sense (C1 to C4) putative translation products were identified in the circular ssDNA genome of GGVA (Fig. 3A; Table 3). A blastp analysis of the predicted proteins of GGVA showed similarities as well as differences with viruses belonging to different genera in the family *Geminiviridae*. For instance, whereas most of the complementary-sense ORFs of GGVA show greater resemblance to begomoviruses, the virion-sense ORFs are more related to the recently characterized Apple geminivirus (AGV [KM386645]) (Liang et al. 2015) whose genus designation is yet to be determined. The ORF V1 is 771 nt in length and its translation product (256 aa; 29.3 kDa) is most identical to the CP of AGV (48% identity; query coverage, 100%; E value, 3e-86). Similarly, the translation product (103 aa; 11.7 kDa) of the 312 nt long ORF V2 of GGVA is most identical to the V2 protein of AGV (52% identity; query coverage, 63%; E value, 1e-18). The predicted CP-encoded nuclear localization signal (NLS) was identified as “NPRKRKSFA” (E value: 0.81).

Unlike the virion-sense ORFs, the complimentary-sense proteins of GGVA are more identical to their homologs in two of the seven currently recognized genera in the family *Geminiviridae*. The translation product of ORF C1 (1,212 nt; 403 aa; 45.1 kDa) is most closely related to the replication-associated protein (AC1) of the begomovirus *Tomato yellow leaf curl Thailand virus* (TYLCTHV-D [AF206674]; 63% aa identity; query coverage, 91%; E value, 6e-179) but its predicted C2 protein (420 nt; 139 aa; 15.6 kDa) is most identical to the transcriptional activator protein (AC2) of *African cassava mosaic virus* (ACMV [X17095]; 36% aa identity; query coverage, 92%; E value, 2e-15), another begomovirus. Similarly, the predicted ORFs C3 of GGVA (429 nt; 142 aa; 16.4 kDa) is most identical to the replication enhancer protein (AC3) of another begomovirus *Indian cassava mosaic virus* (ICMV [Z24758]; 39% aa identity; query coverage, 94%; E value, 1e-26). In contrast to ORFs C1 to C3, the predicted ORF C4 of GGVA (258 nt; 85 aa; 9.9 kDa) shared the greatest resemblance to the C4 protein of *Beet curly top virus* (BCTV [M24597]; 64% aa identity; query coverage, 100%; E value, 8e-29), a member of the genus *Curtovirus*. An alignment of the N-terminal portion of the replication-associated protein sequence of GGVA showed that it also contains the dsRNA-

binding motifs FLTYP (motif I), HLH (motif II), and YIEKD (motif III) that have been reported in other geminiviruses and are implicated in protein conformational changes and viral DNA cleavage in addition to the so-called GRS domain (Fig. 4; Nash et al. 2011).

In pairwise comparisons, the complete genome sequence of GGVA shared maximum identities (50 to 51%) with six begomoviruses (Table 4), its complete CP amino acid sequence was most identical (48%) to corresponding sequences of AGV, while its complete rep protein sequence shared maximum identities (50 to 58%) with AGV, *Beet curly top virus* (BCTV), *Tomato pseudo-curly top virus* (TPCTV), and several begomoviruses (Table 4). Taken together, these results showed that GGVA is a unique novel member of the plant virus family *Geminiviridae* sharing sequence characteristics with members of diverse genera within the family.

Genome organization and molecular properties of GGVA

D-DNA. Like its parental virus, the stem-loop sequence of the intergenic region of the GGVA D-DNA molecule also contains the geminiviral nonanucleotide “TAATATT↓AC” (Fig. 3C). Further analysis revealed that the ~46% deletion in the genome parental virus that gave rise to GGVA D-DNA occurred due to an excision of ORFs C2, C3, and C4, and a near complete (82%) loss of the C-terminal amino acid sequences of ORF C1 corresponding to nucleotide positions 1265 to 2679 on the GGVA genome (Fig. 3B). As a result, only one of the dsRNA-binding motifs (FLTYP) was retained by in the N-terminal portion of the GGVA D-DNA Rep protein sequences. Interestingly, both virion-sense ORFs V1 and V2 were not disrupted (Table 3; Fig. 3B), suggesting that the defective DNA molecule of GGVA could potentially be encapsidated to form isometric particles thus giving rise to the probability of its possible transmission by a yet-to-be-identified vector. Similar observations have been made for defective DNA molecules associated with *Ageratum yellow vein virus* (AYVV) (Stanley et al. 1997) and *Cotton leaf curl virus* (CLCuV) (Liu et al. 1998). In addition, GGVA D-DNA also retained the CP-encoded importin α -dependent NLS, suggesting that it is also capable of being shuttled in and out of the nucleus (Fontes et al. 2004; Rojas et al.

Fig. 3. Genome maps of the genomic **A**, and defective **B**, circular ssDNA molecules of Grapevine geminivirus A (GGVA). Arrows denote the virion-sense (V) and complementary-sense (C) geminiviral genes encoded by each molecule. IR, intergenic region sequences; V1, coat protein; V2, pre-coat protein; C1, replication-associated protein; C2, transcriptional activator protein; C3, replication enhancer protein; C4, host activator protein. **C**, The stem-loop in the IR of GGVA and GGVA D-DNA is depicted with the conserved geminiviral nonanucleotide sequence “TAATATT↓AC” shown in gray arc box (nucleotide position 2899 to 2). Number 1 (gray arrow) indicates position 1 in the viral genome corresponding to the predicted replication origin of the viral DNA.

2001). These results represent the first report of the association of a defective DNA molecule with a grapevine virus.

Phylogenetic relationships between GGVA and other taxa in the family *Geminiviridae* Phylogenetic relationships inferred using aligned complete genome sequences of GGVA,

representatives of the currently recognized genera in the family *Geminiviridae*, as well as yet to be classified members of the family revealed contrasting evolutionary history for the virus. Based on complete virus genome sequences, GGVA formed a distinct cluster sandwiched between AGV and TPCTV on the neighbor-joining

TABLE 3. Genome features of type isolates of Grapevine geminivirus A (GGVA) and its defective DNA molecule (GGVA D-DNA)

Isolate	Genome length (nt)	Protein ID ^a	Length (nt)	Length (aa)	Molecular weight (kDa)	Start	End
GGVA-NP	2,905	Rep/C1	1,212	403	45.1	1,683	2,894
		CP/V1	771	256	29.3	446	1,216
		Ren/C3	429	142	16.4	1,213	1,641
		TrAP/C2	420	139	15.6	1,364	1,783
		V2	312	103	11.7	313	624
		C4	258	85	9.9	2,390	2,647
GGVA D-DNA (NP-4-1)	1,559	IR/NCR	323	NA	NA	1...312	2,895...2,905
		CP/V1	771	256	29.3	446	1,216
		N-terminal Rep/C1	336	111	12.5	1,213	1,548
		V2	312	103	11.7	313	624
		IR/NCR	323	NA	NA	1...312	1,549...1,559

^a Rep, replication-associated protein; CP, coat protein; Ren, replication enhancer protein; TrAP, transcriptional activator protein; IR, intergenic region; and NCR, noncoding region.

Fig. 4. Multiple alignment of the N-terminal portion of Rep protein sequences of Grapevine geminivirus A (GGVA) and other members of the family *Geminiviridae* highlighting conserved motifs I, II, III, and GRS. AGV, Apple geminivirus; BCTV, Beet curly top virus; HrCTV, Horseradish curly top virus; SCTAV, Spinach curly top Arizona virus; BCTIV, Beet curly top Iran virus; TCTV, Turnip curly top virus; ECSV, *Eragrostis curvula* streak virus; WDV, Wheat dwarf virus; MSV, Maize streak virus; PanSV, Panicum streak virus; TPCTV, Tomato pseudo-curly top virus; GRBaV, Grapevine red blotch-associated virus; FbSLSV, French bean severe leaf curl virus; EcmLV, *Euphorbia caput-medusae* latent virus; MMDaV, Mulberry mosaic dwarf-associated virus; CCDaV, Citrus chlorotic dwarf-associated virus; TLCCNV, Tomato leaf curl China virus; ELCV, *Euphorbia* leaf curl virus; ToLCMGV, Tomato leaf curl Madagascar virus; EACMV, East African cassava mosaic virus; TYLCNV, Tomato yellow leaf curl China virus; AYVV, *Ageratum* yellow vein virus; and ToLChAV, Tomato leaf curl Hainan virus.

phylogenetic tree (Fig. 5C). However, it clustered more tightly with AGV in a cluster very distinct from those formed by other geminiviruses but sandwiched between curtoviruses and begomoviruses on the CP amino acid-based tree (Fig. 5A). The rep (C1

protein) based tree showed that GGVA isolates NP and BB represent a distinct genus-level clade sandwiched between clades formed by turncurtoviruses and begomoviruses/AGV (Fig. 5B). Similar topologies were observed on phylogenetic trees constructed using

TABLE 4. Size and range of complete genome and gene-specific amino acid sequences between Grapevine geminivirus A (GGVA) isolate Nagano Purple and GGVA isolate Black Beet (BB) along with published viruses representing the species diversity within the family *Geminiviridae*

Virus ^a	Genus	Host Type	Vector	GenBank accession number	Genome size (nt)	Genome sequence identity (%)	Amino acid sequence identity (%)	
							CP	Rep
GGVA-BB	Unassigned	Dicot	Unknown	KX570609	2,905	97.9	99.2	98.0
AGV	Unassigned	Dicot	Unknown	KM386645	2,932	46.0	47.6	50.8
BCTV	<i>Curtovirus</i>	Dicot	Leafhopper	AF379637	3,038	38.2	12.9	49.7
HrCTV	<i>Curtovirus</i>	Dicot	Leafhopper	U49907	3,080	34.9	14.0	41.3
SCTAV	<i>Becurtovirus</i>	Dicot	Leafhopper	HQ443515	2,860	27.9	13.7	17.9
BCTIV	<i>Becurtovirus</i>	Dicot	Leafhopper	EU273818	2,845	28.1	12.5	16.7
TCTV	<i>Turncurtovirus</i>	Dicot	Leafhopper	KT388064	2,969	36.2	13.7	47.9
ECSV	<i>Eragrovirus</i>	Monocot	Leafhopper	FJ665629	2,746	32.2	12.2	35.3
WDV	<i>Mastrevirus</i>	Monocot	Leafhopper	X02869	2,749	27.4	10.5	21.7
MSV	<i>Mastrevirus</i>	Monocot	Leafhopper	AF329881	2,689	27.0	12.8	22.9
PanSV	<i>Mastrevirus</i>	Monocot	Leafhopper	L39638	2,705	26.4	13.9	15.5
TPCTV	<i>Topocuvirus</i>	Dicot	Treehopper	X84735	2,861	39.1	12.8	50.8
GRBaV	Unassigned	Dicot	Treehopper	JQ901105	3,206	30.0	14.2	25.5
FbSLCV	Unassigned	Dicot	Unknown	JX094280	2,771	27.9	15.0	22.8
EcmLV	Capulavirus ^b	Dicot	Unknown	HF921477	2,650	26.2	14.2	25.3
MMDaV	Unassigned	Dicot	Unknown	KP303687	2,952	29.1	14.4	25.9
CCDaV	Unassigned	Dicot	Whitefly	JQ920490	3,640	27.9	11.5	22.2
TLCCNV	<i>Begomovirus</i>	Dicot	Whitefly	AJ704603	2,733	50.6	11.9	56.6
ELCV	<i>Begomovirus</i>	Dicot	Whitefly	AJ558121	2,746	50.4	14.3	58.0
ToLCMGV	<i>Begomovirus</i>	Dicot	Whitefly	AJ865339	2,775	50.5	12.9	56.9
EACMV	<i>Begomovirus</i>	Dicot	Whitefly	JF909175	2,801	47.3	14.3	53.9
TYLCCNV	<i>Begomovirus</i>	Dicot	Whitefly	KC189893	2,737	49.9	12.6	57.4
AYVV	<i>Begomovirus</i>	Dicot	Whitefly	KC810890	2,759	49.9	15.0	54.7
ToLCHaV	<i>Begomovirus</i>	Dicot	Whitefly	HQ162270	2,740	50.2	13.6	57.1

^a AGV, Apple geminivirus; BCTV, *Beet curly top virus*; HrCTV, *Horseradish curly top virus*; SCTAV, *Spinach curly top Arizona virus*; BCTIV, *Beet curly top Iran virus*; TCTV, *Turnip curly top virus*; ECSV, *Eragrostis curvula streak virus*; WDV, *Wheat dwarf virus*; MSV, *Maize streak virus*; PanSV, *Panicum streak virus*; TPCTV, *Tomato pseudo-curly top virus*; GRBaV, *Grapevine red blotch-associated virus*; FbSLCV, *French bean severe leaf curl virus*; EcmLV, *Euphorbia caput-medusae latent virus*; MMDaV, *Mulberry mosaic dwarf-associated virus*; CCDaV, *Citrus chlorotic dwarf-associated virus*; TLCCNV, *Tomato leaf curl China virus*; ELCV, *Euphorbia leaf curl virus*; ToLCMGV, *Tomato leaf curl Madagascar virus*; EACMV, *East African cassava mosaic virus*; TYLCCNV, *Tomato yellow leaf curl China virus*; AYVV, *Ageratum yellow vein virus*; and ToLCHaV, *Tomato leaf curl Hainan virus*.

^b Tentative genus.

Fig. 5. Unrooted neighbor-joining phylogenetic trees reconstructed from the alignment of the amino acid sequences of **A**, coat protein, **B**, rep protein, and **C**, the full-genome nucleotide sequences of Grapevine geminivirus A (GGVA) and representative members of the family *Geminiviridae*. The trees were constructed using the MEGA6 program (Tamura et al. 2013) and percent bootstrap values (1,000 replicates) are indicated at the branch internodes. AGV, Apple geminivirus; BCTV, *Beet curly top virus*; HrCTV, *Horseradish curly top virus*; SCTAV, *Spinach curly top Arizona virus*; BCTIV, *Beet curly top Iran virus*; TCTV, *Turnip curly top virus*; ECSV, *Eragrostis curvula streak virus*; WDV, *Wheat dwarf virus*; MSV, *Maize streak virus*; PanSV, *Panicum streak virus*; TPCTV, *Tomato pseudo-curly top virus*; GRBaV, *Grapevine red blotch-associated virus*; FbSLCV, *French bean severe leaf curl virus*; EcmLV, *Euphorbia caput-medusae latent virus*; MMDaV, *Mulberry mosaic dwarf-associated virus*; CCDaV, *Citrus chlorotic dwarf-associated virus*; TLCCNV, *Tomato leaf curl China virus*; ELCV, *Euphorbia leaf curl virus*; ToLCMGV, *Tomato leaf curl Madagascar virus*; EACMV, *East African cassava mosaic virus*; TYLCCNV, *Tomato yellow leaf curl China virus*; AYVV, *Ageratum yellow vein virus*; and ToLCHaV, *Tomato leaf curl Hainan virus*.

maximum likelihood and minimum evolution methods (data not shown) thus validating the observed clustering patterns. Similar clustering patterns were observed between genome and protein specific sequence trees derived in this study and corresponding trees generated in other recent studies (Liang et al. 2015; Ma et al. 2015) providing further validity of the results.

GGVA and its defective molecule are graft-transmissible. In bud chip graft transmission assays, GGVA and its co-infecting viruses were successfully transmitted from the NP and BB source onto Cabernet franc (Cf) indicator vines. An indication of the success of the graft transmission assay was the observation of leafroll disease symptoms on leaves of the Cf indicator vines (Fig. 1C and D), as would have been predicted by the HTS identification of leafroll viruses in the NP and BB source vines. Interestingly, the chlorotic ringspot symptom observed on the NP source was reproduced on the Cf indicator in addition to leafroll disease symptoms (Fig. 1C). In contrast, the Cf grafted with BB source expressed only leafroll disease symptoms (Fig. 1D). Cf materials infected from NP and BB source vines also tested positive by RT-qPCR for other viruses originally identified in the respective vines. However, whereas GGVA was detected from Cf on both NP and BB sources, GGVA D-DNA was detected only from Cf on the NP source in agreement with the results of the PCR tests. None of the mechanically inoculated herbaceous indicator plants produced discernible virus-like symptoms and all tested negative for GGVA and GGVA D-DNA by PCR (data not shown), indicating that the virus and its defective DNA are not mechanically transmissible to herbaceous plants. These results show that GGVA and its defective DNA molecule are graft-transmissible.

Validation of the qPCR assay. A qPCR assay was developed for the detection of GGVA. The primers and probe for the assay (Table 1) were designed based on alignments of 11 full-length GGVA genomes derived from multiple grapevine cultivars (Table 5). For assay validation, five 10-fold serial dilutions of DNA extracted from infected tissue samples and undiluted DNA were subjected to qPCR. As shown in Supplementary Fig. S2A, normal amplification curves were obtained for the DNA dilution series difference. In addition, the standard curve constructed based on the plot of the DNA dilutions against corresponding Cq values revealed a linear relationship with a regression coefficient (R^2) value of 0.9996. The primer efficiency (E) value was determined to be 95.22, indicating that

the majority of the target molecules were amplified during the course of the qPCR assay.

Occurrence of GGVA in additional vines from multiple geographical locations. Of the 1,262 samples in the field survey for GGVA, 585 were from the Davis Grapevine Virus Collection (DGVC), 230 were from the USDA National Clonal Germplasm Repository (NCGR) vineyards, 250 were from the FPS Introduction Pipeline, and 197 were from commercial vineyards. The newly developed qPCR assay (Table 1) was used to screen all samples for GGVA. In qPCR tests, 22 vines (1.74%) tested positive for GGVA (Table 5). A breakdown of the positive vines by collection type showed that 0.51% (3 of 585) came from the DGVC, 6.52% (15 of 230) from the USDA NCGR, and 1.6% (4 of 250) from the FPS Introduction Pipeline. None of the 197 vines sampled from commercial vineyards tested positive for GGVA. Further analysis of the 22 positive vines indicated that the vines were originally introduced from six countries (Israel = 3, Japan = 10, China = 4, United States = 1, Hungary = 1, and South Korea = 3) and were derived from 15 grapevine cultivars (Table 5). Although the overall incidence of GGVA-positive vines (1.74%) would suggest a low incidence of the virus, the diversity of sources of the positive vines points to a possible global distribution.

DISCUSSION

It has long been thought that the host range of geminiviruses is limited to herbaceous plants. However, the last few years have witnessed an increase in the number of geminiviruses from woody perennial plant species. The expanding list of geminiviruses characterized from woody host plants includes *Citrus chlorotic dwarf-associated virus* (CCDaV) (Loconsole et al. 2012), *Grapevine red blotch-associated virus* (GRBaV) (Al Rwahnih et al. 2013; Krenz et al. 2012; Poojari et al. 2013), *Jatropha mosaic virus* (JaMV) (Polston et al. 2014), *Mulberry mosaic dwarf-associated virus* (MMDaV) (Ma et al. 2015), and *Apple geminivirus* (AGV) (Liang et al. 2015). With the report of yet another putative member of the family *Geminiviridae*, GGVA, in this study, it is becoming clear that the host range of this economically important plant virus family is more extensive than previously realized. Interestingly, these new groups of geminiviruses are so distantly related to members of the currently recognized genera in the family that they

TABLE 5. Occurrence of Grapevine geminivirus A (GGVA) in a subset of vines from various grapevine germplasm collections maintained by the United States Department of Agriculture (USDA) and Foundation Plant Services (FPS) at the University of California, Davis (UC-Davis), CA

Source	Plants in collection	Total tested	Total positive	Variety	Grape type	Country	Location	Accession number
UC-Davis GVC ^a	788	585	3	Tamar	Table/wine	Israel	ARM VSV 9 V19-21	N/A
USDA NCGR ^b	5,461	230	2	Koshu Sanjaku	Table/wine	Japan	H3 V11, V12	KX570617
			2	Muscat Angel	Table	Japan	H3 V37, V38	N/A
			1	Super Hamburg	Table/wine	Japan	H4 V38	KX570610
			1	Pione	Table	Japan	H17 V30	KX570616
			1	J-167-045	Unknown	China	H42 V51	N/A
			1	Kyoho	Table	Japan	K9 V10	KX570612
			2	Neo-Muscat	Table	Japan	K13 V35, V36	KX570613
			1	Longyan	Table/wine	China	L2 V13	KX570611
			2	Longyan	Table/wine	China	L13 V21, V22	N/A
			1	Scolokertek Kiralynojje	Table	Hungary	K7 V8	KX570618
			1	Pearl of Zola	Table	United States	K13 V38	N/A
			1	Black Beet	Table	South Korea	GH1 N 6 0	KX570609
			1	Nagano Purple	Table	South Korea	GH1 N 6 0	KX570607
FPS New Introduction Pipeline	N/A	250						KX570608 ^c
								KX570615
								KX570614
Commercial vineyards	N/A	197	N/A	N/A	N/A	N/A	N/A	N/A

^a UC-Davis Grapevine Virus Collection.

^b USDA NCGR, U.S. Department of Agriculture National Clonal Germplasm Repository.

^c Accession number for the Nagano Purple GGVA D-DNA genome.

may represent genus-level members of the family. Consequently, there is a need for a comprehensive review of the taxonomy of the family *Geminiviridae* to better understand the evolutionary histories and implications of the discoveries of these perennial plant-infecting viruses. A pertinent issue to be considered is whether woody plant-infecting geminiviruses are ancient relics of the family *Geminiviridae* that coevolved with their hosts. Alternatively, they may be viruses of herbaceous plants that made a host jump to perennial plants; or they may be viruses of wild relatives of woody perennials that have evolved to infect commercial cultivars. Intensive surveys that include sampling of wild relatives and weed species around the vicinities of the infected blocks of orchards and vineyards would help gain some insights into these questions. As more geminiviruses are discovered from woody perennials, thanks to the versatility and sensitivity of the HTS and the RCA technology, as well the culture-independent nature of both technologies, the availability of more sequences would enable a better resolution of the phylogeny and evolutionary history of this family of agriculturally relevant viruses.

The discovery of GGVA by HTS further strengthens the argument for the incorporation of this technology into the grapevine indexing program for the optimal detection of viral pathogens of potential agronomic significance (Al Rwahnih et al. 2015). With the symptomology of GGVA still unclear, its presence would be undetectable by standard biological indexing which relies on symptoms induced by the virus in selected indicator host plants. GGVA would have also escaped detection in standard RT-qPCR assays since these require a prior knowledge of the viral pathogen for the selection and design of virus-specific oligonucleotides. Not surprisingly, all but one (JaMV) (Polston et al. 2014) of the aforementioned woody plant-infecting geminiviruses were discovered via HTS. We hypothesize that more viruses, including those with circular ssDNA genomes, will be discovered and characterized from grapevines especially as the use of HTS technology becomes more routine.

Defective DNAs that are often half the size of the viral genomic components have been reported for several geminiviruses infecting different herbaceous host plants (Patil and Dasgupta 2006 and references therein). To the best of our knowledge, none have been reported for any of the woody host plant-infecting geminiviruses. Therefore, another significant outcome of this study is the discovery of GGVA D-DNA—a natural defective DNA molecule associated with GGVA in cultivar Nagano Purple. We simply refer to this subviral circular ssDNA molecule at this time as defective DNA (D-DNA) due to a lack of evidence so far to suggest that it interferes with the replication of the viral genomic DNA molecule as reported for similar geminiviral defective DNA molecules (Ndunguru et al. 2006; Paximadis and Rey 2001). The genome of GGVA D-DNA is roughly half the size of the GGVA genomic component (Fig. 3A and B). GGVA D-DNA was found to occur naturally in a GGVA-infected grapevine and its molecular analysis revealed that it arose due to deletions in the replication associated proteins of the virus (Fig. 3A and B). Unlike some defective DNAs that contain sequences of the helper virus in addition to unrelated sequences such as those associated with *Ageratum yellow vein virus* (AYVV) (Stanley et al. 1997) and *Turnip crinkle virus* (Simon and Howell 1986), no nonviral sequence was found on GGVA D-DNA. The sequence analysis confirmed the presence of the complete IR, V1, and V2 genes and partial N-terminal C1 protein sequences (Fig. 3B and C), suggesting that GGVA D-DNA could be transreplicated by the rep protein of the viral genomic DNA. While there is presently no direct evidence to support the encapsidation of GGVA D-DNA, the conformity of its size, intactness of its V1 ORF and the conservation of potential encapsidation signals such as the stem-loop region (Qin et al. 1998) provide support for the possibility that this subviral DNA molecule may form virions and could be potentially transmitted by a yet-to-be-determined vector species. Furthermore, GGVA D-DNA could possibly exist between the nucleus and the cytosol since it retained the CP-encoded importin α -dependent NLS

(Fontes et al. 2004; Rojas et al. 2001). Further studies are needed to dissect the functions of the limited genes encoded by GGVA D-DNA and to determine if this subviral DNA molecule is capable of forming virions.

There is a considerable gap in knowledge regarding the biology of GGVA and its D-DNA molecule at this time. To begin with, the viral genomic and subviral DNA molecules were found associated with a chlorotic ringspot symptom in vines of *V. vinifera* cultivar NP and in mixtures with other viruses (Fig. 1A; Table 2) and the chlorotic ringspot symptom was reproduced on the standard grapevine virus indicator cultivar Cf in graft-transmission assays (Fig. 1C). However, no apparent foliar symptoms were associated with GGVA when it was present (without GGVA D-DNA) in cv. BB vines. Therefore, the absence of GGVA D-DNA in cultivar BB vines relative to vines of cv. NP makes it plausible to suggest a link between the ringspot chlorotic symptoms and presence of the defective DNA molecule. However, other factors such as cultivar differences, synergism among co-occurring viruses and viroids, etc. could also have contributed to the observed differences in symptomology between NP and BB. Therefore, additional studies are needed to better understand the etiology of the disease symptoms observed in NP and to decipher the pathological effect of GGVA with or without GGVA D-DNA. Further studies are also required to determine the effect of coinfection of GGVA and GGVA D-DNA on the replication of the viral genomic DNA molecule. The availability of infectious clones for GGVA and GGVA D-DNA would help to dissect the compatible interactions among GGVA D-DNA products, the helper virus, and the grapevine host components. Furthermore, the very limited similarities and phylogenetic relatedness of CP amino acid sequences of GGVA with corresponding sequences of geminiviruses with known vectors (Fig. 5A) makes it impossible at this stage to speculate on a possible insect vector for the virus. Extensive field surveys and periodic monitoring of confirmed infected vineyard blocks could reveal spatial patterns to help determine the nature of a possible vector under field conditions thus helping to narrow down candidates to be included in vector transmission assays.

Limited surveys conducted in the National Clonal Germplasm Repository, the Davis Grapevine Virus Collection, the FPS Introduction Pipeline, and commercial vineyards indicated that although the field incidence of GGVA is low, the virus was detected in multiple grapevine cultivars introduced from six different countries across three continents (Table 5). The identified GGVA-positive vines were propagated from introduced grapevine canes thus suggesting that the virus was introduced via cuttings that originated from infected vines from the different country sources. It is therefore likely that GGVA may have a greater global widespread and that it could have been possibly moved across international boundaries undetected due to its novel nature and a lack of consistent symptomology.

A step toward gaining a better understanding of the aforementioned gaps in the knowledge of GGVA and GGVA D-DNA biological properties is the development of diagnostic assays for the routine detection of both molecules and their prevalence in epidemiological studies. The conventional and qPCR assays (Table 1; Fig. 2) developed in this study will prove valuable in this regard. These assays will be useful in studies to determine the occurrence and prevalence of GGVA and GGVA D-DNA in different grapevine-growing regions of the world; the screening of wild grapevines in riparian habitats for possible alternative hosts of the virus; and the screening of insect species sampled from infected vineyards for potential vectors of the virus.

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