Association of a DNA virus with Grapevines affected by Red Blotch disease in California.

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ABSTRACT

In the Napa Valley of California, vineyards of Cabernet Franc (CF) clone 214, Cabernet Sauvignon clone 337 and Zinfandel clone 1A (Z1A) with grapevines exhibiting foliar symptoms of red blotches, marginal reddening, and red veins, that were accompanied by reduced sugar accumulation in fruits at harvest, were initially suspected to be infected with leafroll-associated viruses. However, reverse transcription-polymerase chain reaction tests were negative for all known leafroll-associated viruses, with the exception of *Grapevine leafroll-associated virus* 2 in Z1A. Metagenomic analysis of cDNA libraries obtained from double-stranded RNA enriched nucleic acid (NA) preparations from bark scrapings of dormant canes on an Illumina platform revealed sequences having distant relationship with members of the family *Geminiviridae*. Sequencing of products obtained by PCR assays using overlapping primers and rolling circle amplification (RCA) confirmed the presence of a single circular genome of 3,206 nucleotides which was nearly identical to the genome of a recently reported Grapevine cabernet franc-associated virus found in declining grapevines in New York. We propose to call this virus 'Grapevine red blotch-associated virus' (GRBaV) to describe its association with grapevine red blotch disease. Primers specific to GRBaV amplified a product of expected size (557 bp) from NA preparations obtained from petioles of several diseased source vines. Chip bud inoculations successfully transmitted GRBaV to test plants of CF, as confirmed by PCR analysis. This is the first report of a DNA virus associated with red blotch disease of grapevines in California.

Keywords: geminivirus, illumina, next generation sequencing, WGA, winegrape

INTRODUCTION

Grape (*Vitis vinifera* L, family *Vitaceae*) production is a major enterprise in the State of California where vineyards account for 90% of the bottled wines in the United States (http://www.wineinstitute.org), and nearly all of the table grapes and raisins produced in the country. The North Coast region of California is a major producer of premium quality wine grapes. Currently, there are about 45,000 acres of wine grapes planted in Napa County which command a highest price per ton in California.

Grape production can be impacted by several pests and diseases. Among grapevine diseases caused by viruses and virus-like agents, leafroll diseases significantly impact vineyard health (13, 28). Currently, there are 11 leafroll-associated viruses that have been recognized as members of the family *Closteroviridae* (29). Recently, Martelli et al. (29) proposed to regroup these viruses into five species; *Grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-3 and GLRaV-4, under the genus *Ampelovirus*, GLRaV-2 in the genus *Closterovirus*, and GLRaV-7 under the new genus *Velarivirus*. Of these, GLRaV-1 and GLRaV-3 are major concerns, in part due to their wider distribution and being vectored by several species of mealy bugs and scales (10, 16, 25, 37). The lone leafroll-associated virus belonging to the genus *Closterovirus*, GLRaV-2, despite its adverse effect on grapevine health and wine quality, is not considered epidemiologically significant because it is not known to be vectored (7, 18).

In grapevines, leafroll disease is recognized by green secondary and tertiary veins and interveinal purple to red discoloration of leaf blades in red fruit varieties or pale green to yellow color in white fruit varieties (13). Diseased grapevines decline slowly and the clusters suffer from berries lacking full color, delayed maturity and reduced sugar (16). Wines produced from grapes affected by leafroll disease are inferior in quality (21, 26).

Some strains of leafroll associated viruses, such as GLRaV-2, and GLRaV-7, can be asymptomatic in nature (3, 31, 38). For example, GLRaV-2 strain RG is asymptomatic in cultivar Red globe table grapes and Cabernet Franc clone 01, the indicator host of most leafroll-associated viruses including the type strain of GLRaV-2 (38). In addition to leafroll, several red leaf diseases involving degenerative symptoms on woody cylinders of grapevine trunks have been recognized in grapevines in California (2, 4, 38). In a few cases, the etiological agent remains unknown despite arduous investigations (2, 4). Even so, during the past decade, much attention has been given to the characterization and recognition of viruses of importance in the context of plant health and berry production. Concomitantly, several new grapevine viruses have been described in recent years in symptomatic and asymptomatic grapevines (1, 2, 5, 11, 24, 30, 40). Application of metagenomic analysis aided by the next generation sequencing technology has been pivotal in the discovery of some of the new viruses (2, 33).

Recently, vineyards in Napa County planted to red fruited varieties were observed to develop leafroll-like symptoms (8). However, symptoms consisted of red blotches on leaf blade and leaf margin with red secondary and tertiary veins. Fruits on affected grapevines measured about 2.4° Brix lower than fruits from grapevines with green canopies (8). Herein, we describe the association of a DNA virus, recently described as Grapevine cabernet franc associated virus (GCFaV) (23), in grapevines affected by red blotch disease.

MATERIALS AND METHODS

Vineyard visits and specimens. In fall 2009, three commercial vineyards planted to Cabernet Franc clone 214 (CF214) on rootstock 101-14 at Oakville, Cabernet Sauvignon clone 337 (CS337) on roostock O39-16 at Rutherford, and Zinfandel 1A (Z1A) on roostock AxR#1 at

Calistoga, and an experimental vineyard of UC-Davis planted to Cabernet Sauvignon 7 (CS-7) on rootstock 101-14 at Oakville, all located in Napa County, CA, containing several grapevines with leafroll-like symptoms were monitored. Several of the diseased grapevines had been marked by the vineyard managers and these grapevines had tested negative for leafroll viruses with the exception of GLRaV-2 in Z1A (virus assays were performed by commercial virus testing service providers in California). At these sites, we categorized foliar symptoms, cane growth and evidence of any trunk markings. Woody canes were harvested in November 2010 from single symptomatic grapevines of CF214, CS337 and Z1A and stored at 4 °C. A control sample of dormant canes was also collected from Pinot Noir clone 91 (PN) grapevine in the UCD vineyard maintained by the Foundation Plant Services.

q-RT-PCR assays for grapevine viruses. Bark scrapings of dormant canes were obtained, ground using a coffee grinder (Mr.Coffee, Sunbeam products Inc., Boca Raton, FL) and stored at -80 °C freezer until processed. Total RNA was extracted from a subsample of ground bark scrapings of symptomatic CF214, CS337 and Z1A sources using RNeasy plant mini kit (www.quiagen.com) and analyzed for grapevine viruses by qRT-PCR using TaqMan probes on ABI 7900 HT Fast real time PCR system (www.invitrogen.com) as described previously (19, 32). Taq®Man probes used in the assays were targeted to leafroll-associated viruses and their strains, including GLRaV-1, GLRaV-2, GLRaV-2RG, GLRaV-3, GLRaV-4, GLRaV-7, GLRaV-4 strains 9 and Car; maculavirus *Grapvine fleck virus*; nepoviruses *Grapevine fanleaf virus*, *Tobacco ringspot virus*, and *Tomato ringspot virus*; vitiviruses *Grapevine virus* A (GVA), GVB, and GVD; and foveavirus *Grapevine rupestris stem-pitting associated virus* (GRSPaV).

Double stranded RNA (dsRNA) extraction and deep sequencing of nucleic acid extracts from bark scrapings. Nucleic acid enriched for double stranded ribonucleic acid (dsRNA) was extracted from 30 g ground bark scrapings as per Routh et al. (34), but without the DNase and RNeasy treatment. Complementary DNA (cDNA) libraries were prepared using the SuperScript® II Reverse Transcriptase kit (www.invitrogen.com) primed with random hexamers (300 ng/µl, www.invitrogen.com) and amplified with GenomePlex® complete whole genome amplification kit (www.sigmaaldrich.com) as per manufacturer's instructions, but without the fragmentation step. The amplified DNA preparation was cleaned using a PCR cleanup kit (www.sigmaaldrich.com) and the DNA quality was verified as described previously (2).

Sequence data was generated by Eureka Genomics Inc (www.eurekagenomics.com) using an Illumina Genome Analyzer IIx (www.illumina.com). From each sample, an Illumina indexed library was prepared from 1 µg of cDNA for single-end, multiplexed sequencing on Genome Analyzer IIx. Briefly, the cDNA was fragmented, end repaired, A-tagged, ligated to adaptors, size-selected and enriched with 18 cycles of PCR. Sample preparation was performed according to a proprietary method of Eureka Genomics Inc and Illumina's Multiplexing Sample Preparation Guide. For each library, two lanes of single-end 36 cycle reads of sequence data were generated and the resulting sequence reads were filtered using the default parameters of the Illumina QC pipeline (CASAVA) and demultiplexed. Reads from each of the samples were used to generate de-novo assembly using algorithm Velvet (39) and data analysis was done at http://www.ncbi.nlm.nih.gov/ using BLASTN for highly similar (megablast) and somewhat similar nucleotide sequences (blastn) and BLASTX (6). The contigs used for these analyses were at least 100 nucleotides in length.

Detection of a DNA virus in grapevines exhibiting red blotch symptoms by PCR. The sequences of contigs from CF214, CS337, and Z1A were subjected to multiple sequence alignment using CLUSTALW (http://www.genome.jp/tools/clustalw/) and two primers, GVGF1 (5'-CTCGTCGCATTT GTAAGA -3') and GVGR1 (5'-ACTGACAAGGCCTACTACG-3'), were designed based on the identical regions in the three contigs. Nucleic acid (NA) extracts were prepared from leaf petioles of grapevines with red blotch symptoms in Napa County using a MagMAX™-96 Viral RNA Isolation Kit (www.invitrogen.com) as per manufacturer's protocol. Amplification of the target was achieved by PCR using primers GVGF1 and GVGR1 and GoTaq (www.promega.com) on an Eppendorf EP Mastercyler S (www.eppendorf.com). Thermocycling conditions included one cycle of 94 °C for 2 min; 35 cycles of 94 °C for 30 sec, 60 °C for 30 secs, and 72 °C for 1 min; and one cycle of 72 °C for 5 min. The amplified products were analyzed by agarose gel electrophoresis using Tris-acetate-EDTA (TAE) buffer and sequenced using GVGF1 and GVGR1 primers by Sanger sequencing at UC-Davis sequencing facility (http://dnaseq.ucdavis.edu).

Determination of the genome of the virus associated with grapevines affected by red blotch disease. Nucleic acid extract from three diseased source grapevines was subjected to isothermal rolling circle amplification (RCA; 17) using the Illustra TempliPhi 100 amplification kit (www.gelifesciences.com). A 2 µl aliquot of the total nucleic acid extract was added to 5 µl sample buffer, denatured for 3 min at 95 °C and cooled to room temperature. After adding 5 µl reaction buffer and 0.2 µl enzyme mix, the reaction mix was incubated overnight (18–20 h) at 30 °C and stopped by incubation at 65 °C for 10 min. Two µl aliquots of the RCA products in a 10 µl volume were digested separately by restriction enzymes *Bam*H I, *Eco*R I and *Pst* I (www.neb.com) for 2 h and the digested products were analyzed by electrophoresis on a 1%

agarose gel in TAE buffer. Subsequently, the RCA products were cleaned using Zymoclean DNA cleaning kit (www.zymoresearch.com) and sequenced.

The full genome of the DNA virus was also amplified using a Phusion High-Fidelity DNA polymerase (www.neb.com) and two overlapping primers GVGA-01 (5'-CATATCTGC AGGAATCGC-3') and GVGA-02 (5'-CAATCTGCAGTTCTAGTGAAAG-3') on ABI 9800 Fast Thermal Cycler. The thermocycling conditions used included one cycle of 98 °C for 30 secs; 35 cycles of 98 °C for 10 sec, 52 °C for 30 secs, and 72 °C for 90 secs; and one cycle of 72 °C for 7 min. The amplified product was purified using Qiaquick PCR purification kit (www.qiagen.com) and the nucleotide sequence was determined by primer walking on both strands starting with the primers GVGA-01 and GVGA-02.

Graft transmission assays. Shoots from source grapevines of CF214, CS337 and Z1A were obtained in September 2011 and after confirmation by PCR, the bud chips were grafted onto test plants of CF01 maintained in a greenhouse. Nucleic acid preparations were obtained at 4 and 11 months post-inoculation from leaf petioles distal to the bud grafts, and at 11 months post-inoculation from root tips, and PCR assays were conducted as described above.

RESULTS

Symptoms of grapevine red blotch. Three commercial vineyards located at Calistoga, Oakville, and Rutherford in Napa County CA, and the Oakville Experimental Vineyard of UC-Davis were inspected during fall seasons of 2009 to 2011. Disease symptoms consisted of leaves with irregular red blotches, red veins, and occasional marginal reddening (Figure 1). The basal leaves were more symptomatic than the middle leaves and the terminal leaves generally remained free from symptoms (Figure 1D). The leaf margins looked normal except in case of

Z1A where some rolling down of leaf margin was evident. The affected vineyards included plantings of CF214 at Oakville, CS07 at Oakville Experimental vineyard of UC-Davis, CS337 at Rutherford and Z1A at Calistoga.

qRT-PCR assay for known grapevine viruses. Total RNA preparations from sources of CF214 and CS337 were tested for grapevine viruses using qRT-PCR assays employing Taq®Man probes for five leafroll-associated viruses, one maculavirus, three nepoviruses and three vitiviruses. Both sources of disease samples tested positive for only GRSPaV. All positive controls and negative controls reacted in the expected manner.

Deep sequencing. Once the qRT-PCR assays using TaqMan probes failed to indicate the presence of any of the 15 known grapevine viruses, metagenomic analysis was attempted on the cDNA libraries prepared using dsRNA fractions from CF214, CS337 and Z1A, not treated with nucleases. The Illumina sequence reads varied from 6.8 to 8.1 million among the cDNA libraries prepared from three diseased sources and healthy PN (Table 1). Of these, roughly, 30% were unique reads and the total sequence data generated from each library ranged from nearly 247 Mb to 292 Mb.

The longest contig assembled was comprised of 16,430 nucleotides from the cDNA library from source Z1A. Subsequent bioinformatics analysis indicated that this contig sequence was 99% identical to a GLRaV-2 sequence in the NCBI database (www.ncbi.nlm.nih.gov). The second longest contig from Z1A was 3,242 base long and had very limited homology with *Chickpea chlorotic dwarf Syria virus* (CCDSV; 36) in BLASTN analysis. A 3,224 bases long contig from source CS337 also showed very limited homology with CCDSV. In contrast, a slightly shorter contig, 1711 bases, from source CF214 showed distant relationship with *Mesta yellow vein mosaic virus,* but only in BLASTX analysis. The BLASTN analysis also revealed

the presence of GRSPaV in libraries from the sources CF214 and CS337, but not from the sources Z1A and PN, consistent with the RT-PCR data. However, these two assembled contigs, 4304 and 5638 n, respectively, shared ~79% identity between them thus representing two genetically diverse strains of GRSPaV. Sequences of several mycoviruses, belonging to the families *Chrysoviridae* and *Totiviridae*, were also found, but not in the DNA libraries of all three source grapevines with red blotch symptoms. Mycovirus sequences were also found in PN which did not show any red blotch symptoms.

Detection of a DNA virus in grapevines from red blotch affected vineyards.

Nucleotide sequences of three contigs from CF214, CS337 and Z1A which showed limited relationship with geminiviruses were aligned using CLUSTALW and two primers were designed to amplify a product of 557 bp. Initial PCR analysis amplified a product of expected size from NA preparations prepared from diseased sources of CF214, CS337, and Z1A and also from cDNA libraries used for deep sequencing (Figure 2A). Subsequent PCR assays conducted on NA preparations from three symptomatic CF214 plants in Oakville vineyard indicated the presence of the virus in diseased CF214 plants, but not in healthy CF01 plants from UCD vineyard (Figure 2A). Nucleotide sequence of the amplified products matched the contig sequences generated by Illumina sequencing. Several symptomatic grapevines close to the source vines were also confirmed as infected by the virus in PCR analysis (data not shown).

In July 2012, in a limited survey in an experimental vineyard of Cabernet Sauvignon at the Enology and Viticulture Station of UC-Davis at Oakville, NA preparations of leaf petioles from twenty grapevines were assayed by PCR before the onset of symptoms. Ten of these vines were from the end of a block with disease incidence close to 100% in fall 2011 and the other ten were from the end where the disease incidence was low. After the completion of PCR assay, the

vineyard was visited in Sept 2012 and the disease status of tested vines was recorded. All vines that were positive for the virus in PCR (Figure 2b) were symptomatic while those negative for the virus were symptomless. These results confirmed that a DNA virus was associated with grapevines showing red blotch symptoms and the virus was tentatively called 'Grapevine red blotch-associated virus (GRBaV)'.

The new DNA virus genome resembles a monopartite geminivirus. The full genome sequence of the GRBaV was determined from a 3.2 kbp PCR product amplified from NA from symptomatic CF214 grapevine using two overlapping primers GVGA-01 and GVGA-02. The amplified product was initially sequenced using the primers used for amplification and subsequently by genome walking using several additional primers. The circular genome comprises 3,206 nucleotides with a nonanucleotide sequence typical of geminiviruses. The Intergenic Region (IR) contained the characteristic inverted repeat capable of forming a stem-loop structure. Highly conserved nona-nucleotide sequence (TAATATTAC) was also present within the loop of the IR similar to those found in the IR of three representative geminiviruses (Figure 3A). Predictions using Simvector 4.6 (www.premierbiosoft.com) indicated six open reading frames (ORFs), V2 (292 to 807), V1 (710 to 1384) and V3 (1365 to 1736) in sense orientation and C2 (1898 to 2332), C1 (2250 to 3044) and C3 (2408 to 2890) in complimentary orientation (Figure 3B). In BLASTP analysis, the predicted amino acid sequence of V2 had no relationship with virus encoded proteins in the public domain database (www.ncbi.nlm.nih.gov), but the V1 gene was predicted to encode the capsid protein which had 28% identity (46/165) with *Mesta yellow vein mosaic virus* (GenBank:JX242516). The C1 and C2 ORFs had the capacity to encode for the replication associated proteins sharing homology with C1 and C2 of

Srilankan Cassava mosaic virus (GenBank:CAI64619) and *Miscanthus streak virus*, respectively (GenBank:BAA25579).

While these studies were in progress, BLAST searches showed that GRBaV found in grapevines with red blotch disease was nearly identical to Grapevine cabernet franc-associated virus (GCFaV) found in grapevines in New York State (GenBank:JQ901105). While the nucleotide sequences of the virus isolates CF214-1 and Z1A-1 were nearly identical to GCFaV, the sequence of the virus isolate from CS337-1 (3207 nucleotides) shared 92.7% identity with GCFaV.

To determine if the GRBaV genome is monopartite or bipartite, the circular nucleic acid genome was amplified using RCA. The amplified products, when digested by restriction enzymes *Bam*H I, *Eco*R I and *Pst* I and separated by agarose gel electrophoresis, revealed restriction patterns that could be expected from *in silico* analysis of the nucleotide sequence from full length PCR product obtained using overlapping primers GVG01 and GVG02. The *BamH* I digest produced two fragments of ~1.6 kb from RCA products of sources CF214 and Z1A, but not of source CS337. The *Pst* I digest produced a monomer of 3.2 kb from RCA products obtained from all three sources (Figure 4). Apparently, two point mutations in the virus sequence from CS337 had eliminated the *BamH* I sites. Digestion by *EcoR* I produced three fragments and their migration pattern matched those of 680, 1,140 and 1,386 bp fragments predicted by *in silico* analysis of the sequence of CF214-1. Taken together, restriction fragment analysis using three restriction enzymes indicated that the virus genome is monopartite in nature. The complete sequences of CF214-1, derived from a full-length amplified product, CS337-1 and Z1A-1, derived from sequence obtained by Illumina sequencing, are available as GenBank accessions KC896623, KC896624 and KC896625, respectively.

Graft-transmission assays. Graft transmissibility of the DNA virus was assessed by chip bud inoculations using bud chips from diseased sources of CF214, CS337 and Z1A onto five CF01 test plants per source. The grafted plants were examined by PCR analysis at 4 and 11 months post inoculation. At four months, the virus was not detected NA extracts from petiole samples taken from the leaves distal to the grafted area. However, bark samples close to the graft site tested positive for the virus (data not shown). At 11 months post inoculation, petioles from some leaves distal to the graft sites of inocula sources of CF214 and CS337 tested positive in PCR assays (Table 2). Also, roots of most test plants grafted with all three inocula sources were positive. During the one year observation period, none of the infected test plants developed symptoms of red blotch disease.

DISCUSSION

Grapevine represents one of the most widely grown clonally propagated perennial crops in the world. In many parts of the world, vineyards are established on clonally propagated rootstocks. Before quarantine protocols were established, unregulated movement of propagation materials has contributed to spread of several grapevine viruses. In some instances, the presence of a virus (latent in scion), when propagated on different rootstocks and/or grown in widely different climatic regions served to induce graft incompatibility reactions (14, 15, 38) resulting in red leaf symptoms and rapid decline and death of grapevines. Quite often, the red leaf symptoms are distinct from the leafroll symptoms caused by the members of the genera *Ampelovirus* and *Closterovirus* (family *Closteroviridae*) (4, 38).

In Napa Valley, a new red leaf disease was recognized and later named 'grapevine red blotch disease' (8). Symptomatic grapevines showed leaves with red veins often accompanied by red blotches (Figure 1). While symptoms such as red veins are occasionally seen in leaves suffering from physical damage or stress, red primary veins are not seen in grapevines showing leaf roll disease. Molecular assays for known grapevine viruses were largely negative and a few positives did not correlate with assays of multiple collections. Hence, we proceeded to make cDNA libraries using dsRNA enriched NA fractions from diseased source vines of CF214, CS337 and Z1A, planted in separate vineyards. During NA preparations, RNAse and DNAse treatments were deliberately avoided to include viruses with DNA genomes, such as *Grapevine vein clearing virus* (40).

In metagenomic analysis, among the contigs generated from Illumina reads, a fairly long sequence of GLRaV-2 was found in the library from source Z1A. Contigs of two genetically different GRSPaV, one each from sources CF214 and CS337 were also revealed. These sequences shared 80% identity over 4,300 n length. Contigs of several mycoviruses were also encountered, but these were not seen in all three libraries from diseased sources. Detailed bioinformatics analysis of three contigs, one each of sources CF214, CS337 and Z1A, indicated a weak relationship at the aminoacid level with proteins encoded by geminiviruses. However, the relationship with the four recognized genera (*Mastrevirus*, *Curtovirus*, *Begomovirus* and *Topocuvirus*) of the family *Geminiviridae* was very poor. These results established that the DNA virus present in diseased grapevines was the putative agent of red blotch disease, and hence we proposed to call this virus 'Grapevine red blotch-associated virus (GRBaV)'. While this manuscript was in preparation, GCFaV, tentatively grouped under the family *Geminiviridae*, was reported from grapevines in New York State (23). Bioinformatics analysis revealed that GRBaV

was closely related to GCFaV, and GRBaV isolates CF214-1 and Z1A-1 were nearly identical to GCFaV. However, GRBaV isolate CS337-1 was only 93% identical to GCFaV and thus represents a genetically different strain. Incidentally, the New York isolate was obtained from declining grapevines of Cabernet Franc, but no other biological information is known (23).

We next investigated if the GRBaV found in deep sequencing was also present in the source vines that had been marked in fall 2010 when dormant canes were collected for metagenomic analysis. The three contigs generated from Illumina reads of sources CF214, CS337, and Z1A, with lengths of 1,711, 3,224, and 3,342 nucleotides, respectively, were aligned using CLUSTALW, and two primers, GRBVF1 and GRBVR1, were designed to amplify a 557 bp product. These primers amplified a product of expected size from the cDNA library samples submitted for deep sequencing and also from NA obtained from leaf petioles of diseased source vines. Sequences of the amplified products were verified to be identical to the virus sequences obtained by deep sequencing.

Graft-transmissibility is a key feature of plant viruses in establishing disease etiology (35). Transmission of GRBaV was verified by grafting chip buds of three diseased source vines on test plants of CF01 maintained under greenhouse conditions. Systemic movement of the virus into the leaves distal to the grafted sites was detected at 11 months after inoculation by PCR assays (Table 2). However, the grafted plants did not show any of the red blotch symptoms during the one year postinoculation period.

The full genome of GRBaV was determined by sequencing a 3.2 kb product obtained by PCR amplification using two overlapping primers, GVG01 and GVG02, as well as from amplified products obtained from RCA. The complete sequence of the GRBaV isolate CF-214-1 from CF214 was 3,206 nucleotide long. This length was identical to that of GCFaV and slightly

bigger than the 3,080 nucleotide long genome (of *Horseradish curly top virus* (Genus *Curtovirus*, Family: *Geminiviridae*), a monopartite geminivirus (20).

Recently, another new DNA virus with a genome size of 3.64 kb was found associated with citrus chlorotic dwarf disease (27). Based on the genome organization, the new virus was described as a geminivirus, and was provisionally named Citrus chlorotic dwarf-associated virus (CCDaV). Citrus chlorotic dwarf disease has been known for several decades and the causal agent is known to be transmitted by bayberry whitefly (*Parabemisia myricae* family: *Aleyrodidae*) (22). Both monopartite and bipartite members of the genus *Begomovirus* of the family *Geminiviridae* are known to be transmitted by sweetpotato whitefly (*Bemicia tabaci*). Restriction fragment analysis of RCA amplified products indicated the presence of only one circular DNA molecule in three diseased sources, indicating that GRBaV genome is comprised of a single circular DNA molecule, monopartite, much like CCDaV (Figure 4). From research on sap-transmissible bipartite geminiviruses, it is known that geminiviruses have constraint on the size of the individual DNA components (9, 12). It will be interesting to study how monopartite DNA viruses such as CCDaV and GRBaV could overcome this constraint.

This study has provided evidence for the association of a monopartite DNA virus with grapevine red blotch disease. The PCR assay developed here will be useful in future studies on the incidence, spread, and epidemiology of GRBaV, and to assess the effect of this virus on vineyard health.

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473

Table 1. Quality of sequence reads obtained by Illumina sequencing of amplified cDNA library prepared using dsRNA fractions from red blotch diseased grapevines.

Grapevine cultivar	Total number of reads	Number unique	% Unique	Sequence (Mb)
Cabernet Franc 214[1]	8,131,042	2,556,465	31.4%	292
Cabernet Sauvignon337[1]	6,877,920	2,205,584	32.0%	247
Zinfandel 1A[1]	7,961,669	2,518,810	31.6%	286
Pinot noir 91[2]	7,008,854	2,021,920	28.8%	252

[1] Diseased source plants from commercial vineyards in Napa County

[2] Healthy control from a vineyard maintained by the Foundation Plant Services, UC-Davis.

Table 2. Detection of Grapevine red blotch-associated virus by polymerase chain reaction in Cabernet Franc test plants graft inoculated with chip buds from grapevines showing red blotch symptoms.

			No. GRBaV Positive	
Status	**Cultivar**	**No. Tested**	**Petioles**	**Roots**
a) Symptomatic	Cabernet Franc 214	5	4	2
	Cabernet Sauvignon 337	5	2	4
	Zinfandel 1A	5	0	4
b) Healthy	Cabernet Franc	3	0	0
c) Ungrafted control		3	0	0

Figure Legends:

Figure 1. Symptoms of grapevine red blotch disease on leaves of Cabernet Franc Clone 214 (A) and Cabernet Sauvignon Clone 7 (B) in fall. Red secondary and tertiary veins of a leaf from affected Cabernet Franc grapevine (C). Basal leaves on the shoots of a mature Cabernet Franc clone 214 grapevine showing red blotch symptoms in fall (D).

Figure 2. Detection of Grapevine red blotch-associated virus using primers GVGF1 and GVGR1 in grapevines. Panel A. Detection of the virus in symptomatic source vines used for deep sequencing. Lane 1; kb plus ladder (Invitrogen Inc, Carlsbad, CA), lanes 2 to 4, healthy Cabernet Franc plants from greenhouse; lanes 5 to 7, symptomatic source vines CF214, CS337 and Z1A; lane 8, DNA library used for deep sequencing; lane 9 water. Panel B. Detection of GRBaV by PCR in a vineyard planted with Cabernet Sauvignon at the UCD Enology & Viticulture Station at Oakville in July 2012 before the onset of symptoms. Lane labels M=1kb DNA ladder (Invitrogen Inc., Carlsbad, CA); 1 to 20, nucleic acid extracts from 20 plants; G=grapevines with green canopy in 2011; R= grapevines with red blotch symptoms in 2011; + = positive control as above; - = healthy CF01; W=water control.

Figure 3. The intergenic region (IR) showing nonanucleotide sequence of Grapevine red blotch-associated virus (GRBaV) aligned with three geminiviruses, *Bean yellow dwarf virus* (BeYDV; DQ458791.1), *Chickpea chlorosis virus*-B (CpCV-B;GU256531.1), and *Sri Lankan cassava mosaic virus* (SLCMV-A, AJ890228) (A) and circular genome of GRBaV (B) showing six open

519 reading frames. The nonanucleotide sequence containing the origin of replication is in bold (A).
520 The origin of replication is marked with a downward arrow. The underlined sequences are
521 complimentary and form the stem.
522
523 Figure 4. Restriction analysis of the amplified products obtained from rolling circle
524 amplification of nucleic acid extracts from red blotch diseased grapevines of Cabernet Franc
525 (CF214), Cabernet Sauvignon (CS337) and Zinfandel (Z1A). Lanes at both ends are 1 kb DNA
526 ladder from Invitrogen Inc., Carlsbad, CA. B= *BamH* I, E= *EcoR* I; P=*Pst* I.
527
528

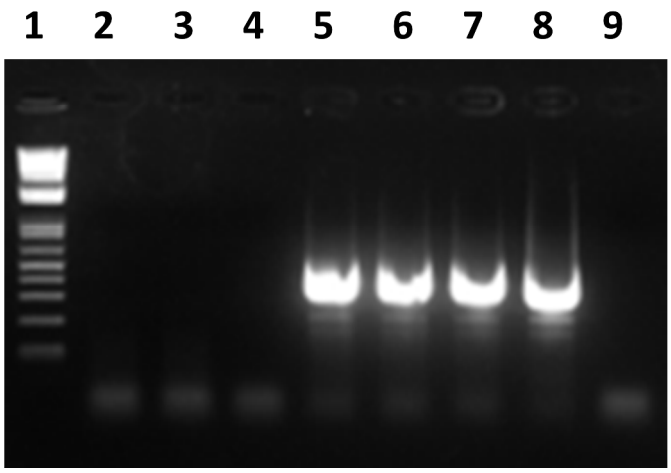
529

530

531 **Figure 1.**

532

A.



B.

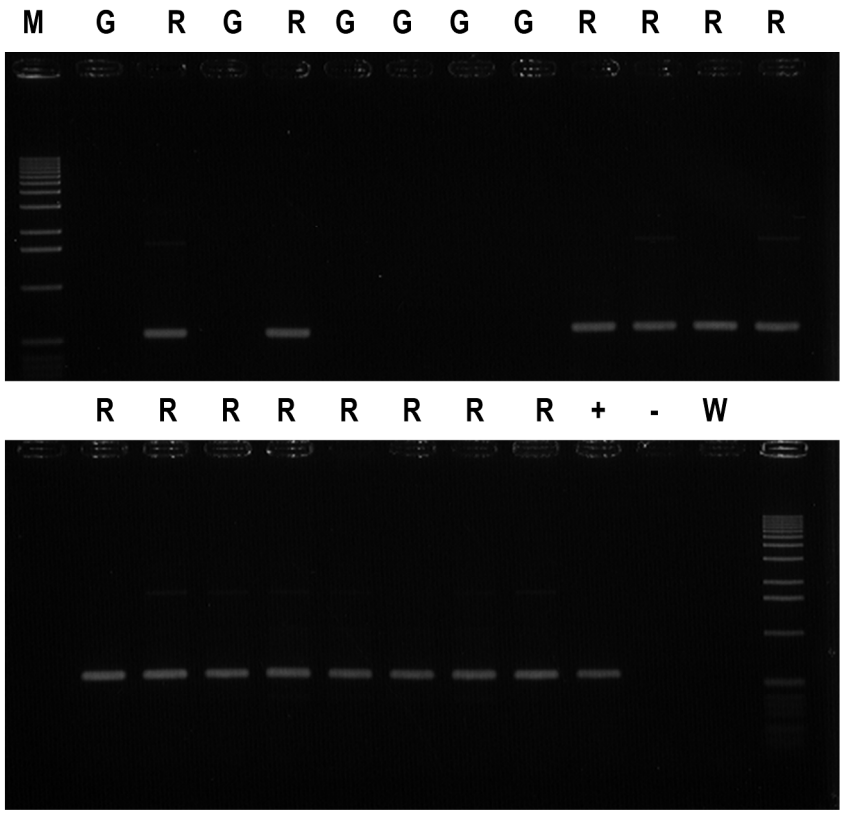


Figure 2.

A

```
GRBaV      TTGTATTGAAGTGCCATATAATATTACTGGCACTTCCCATGGTACGTGGT
BeYDV      GGGGGCCCACGCCGAATTTAATATTACCGGCGTGGCCCCCCCTTATCGCG
CpCV-B     GGGGCGCACTCGCTGATTTAATATTACCAGCGAGTGGCCCCCACGCGAGC
SLCMV-A    GCTAAAGCGCCATCCGTATAATATTACCGGATGGCCGCGCCCCCCGCTTT
```

B.

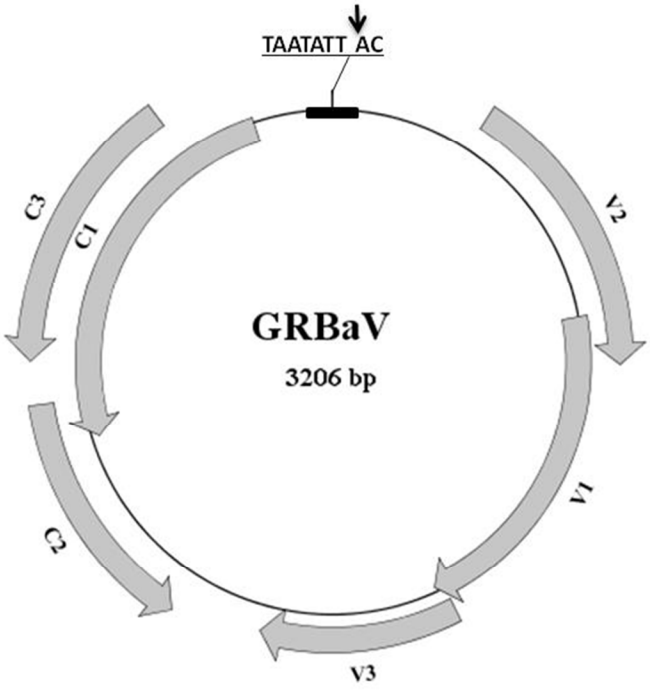


Figure 3.

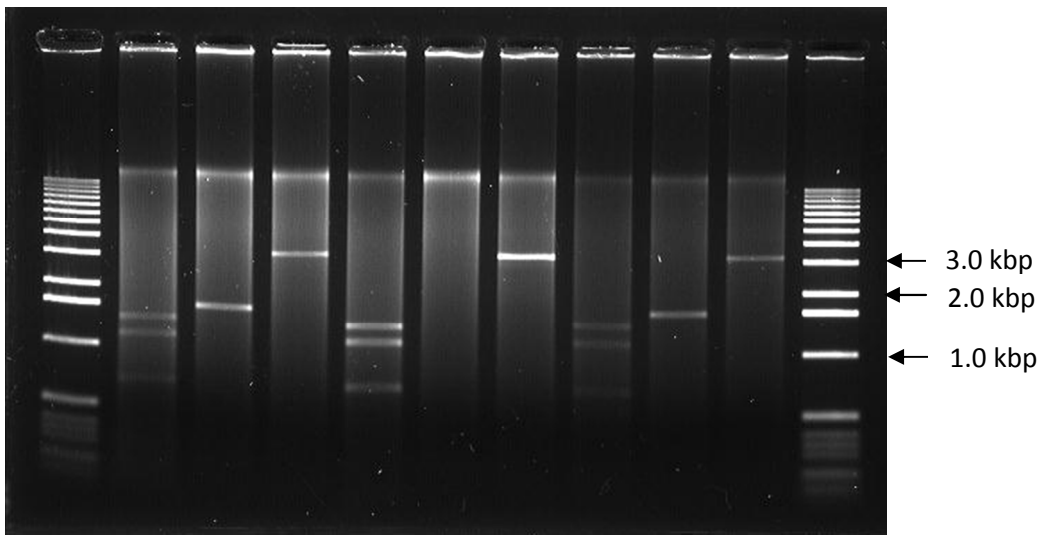


Figure 4.