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Biological, molecular, and serological studies of a novel strain of grapevine leafroll-associated virus 2

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Abstract In California, a novel closterovirus was detected in "Redglobe" grapevine, associated with graft incompatibility and given a trivial name "Grapevine rootstock stem lesion associated virus (GRSLaV)." The biological properties of the putative virus were ascertained when asymptomatic yet infected Redglobe scion buds were graft-inoculated onto test plants of Cabernet Sauvignon propagated on 18 different rootstocks. It proved lethal on test plants growing on rootstocks 1616C, 5BB, 5C, 3309C, and 1103 P, whereas latent infections occurred on the remaining scion-rootstock combinations. In contrast, GLRaV-2 type (type strain) produced only typical leafroll symptoms. In a different experiment, GLRaV-2 type was successfully sap-transmitted to *N. benthamiana*, whereas sap transmission of GRSLaV was unsuccessful. Double-stranded RNA was extracted from infected Redglobe grapevines, cloned, sequenced, and determined a genome length of 16,527 nucleotides. Computer-assisted analysis of open-reading frames (ORFs) revealed a genome organization typical of monopartite viruses in the genus *Closterovirus* with nine ORFs (range 71–79% identity) with GLRaV-2 type, the closest similar virus species within the family *Closteroviridae*. Also the 3′-UTR of GRSLaV consisted of 223 nucleotides with an extended oligo(A) tract similar to that of GLRaV-2 type, *Beet yellow stunt virus*, and *Beet yellows virus*. Recombinant GRSLaV coat protein was expressed in *E. coli*, purified, and immunized a rabbit to produce polyclonal antiserum. Serological data matched the molecular data, whereby exposed plant tissue extracts of grapevines infected by both viruses (GRSLaV and GLRaV-2) reacted positively with homologous and heterologous viral antisera but not with healthy grapevine extracts in ELISA and Western blot tests. Based on the comparative sequence data and shared antigens, GRSLaV is now considered a strain of GLRaV-2 and redesignated as *Grapevine leafroll associated virus*-2 Redglobe (GLRaV-2RG). Primers specific for GLRaV-2RG were developed, which did not amplify GLRaV-2 type strain. When both sets of specific primers were used in assays of different grapevine collections, the incidence of the respective viruses varied considerably, e.g., 1.7 and 13.5%, respectively, for GLRaV-2RG and GLRaV-2 type.

Keywords Redglobe grapevine · *Closteroviridae* · RT–PCR · Genome sequencing · Recombinant coat protein · Graft incompatibility

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Introduction

Grapevine (*Vitis vinifera* L.) is prone to be infected by many different pathogens that shorten its productive life-span worldwide. Viruses are among the most significant of these pathogens and more than 60 different grapevine viruses and graft-transmissible agents have been reported [1–4]. Apart from Grapevine leafroll associated virus 8 (GLRaV-8) that has erroneously been established as a virus

species [5], there are now 11 serologically distinct viruses associated with leafroll disease in grapevines [6–9] and all are members of the family *Closteroviridae* [1].

The type strain of GLRaV-2 (GLRaV-2type) was first reported by Gugerli et al. [10] and was mechanically transmitted to *Nicotiana benthamiana* [11, 12]. Later, 95% of the virus genome was sequenced by Zhu et al. [13] and the sequences were deposited in the GenBank under accession number AF039204.

Following the initial discovery of GLRaV-2 Redglobe strain (formerly named Grapevine rootstock stem lesion associated virus, GRSLaV), other variants of the virus were reported and molecular approaches were used for their identification. For example, GLRaV-2 isolates, such as ''94/970'' and ''93/955'' from *V. vinifera* cv. ''Muscat of Alexandria and hybrid LN-33'' were reported, respectively [12] and the latter isolate was found to have the same identities as Redglobe strain [14]. Furthermore, other distinct strains of GLRaV-2 were found including, WC from *Vitis vinifera* cv. Waltham Cross [15], Alfie virus from New Zealand [16], and GLRaV-2BD from Italy [17] and all were different from PN 93/955 and Redglobe [15].

Specific antibodies are available for identification and detection of the majority of viruses causing leafroll disease in grapevines. Use of purified virus preparation obtained from grapevine tissues for antiserum production has been problematic because of low-virus titer. Nevertheless, viral coat proteins expressed through recombinant DNA technology [18–24] have been used to produce virus-specific antibodies, e.g., to GLRaV-2 and -3 [18, 23]. Serology has been used to distinguish between some of the GLRaV species, for example, between GLRaV-2 and -6 and also between GLRaV-4, -5, and -9 [7, 25, 26].

Herein, we report the collective research studies on GLRaV-2RG regarding biological and serological properties, molecular characterization, comparative genome analysis, and virus incidence in commercial vineyards in California.

Materials and methods

Virus sources and bioassays

A source of GLRaV-2 belonging to Pinot Noir group described by Jarugula et al. [27] (type strain) and an own-rooted grapevine of *Vitis vinifera* cv. Red Globe, a source of GLRaV-2RG, were used for biological assay [28] on test plants of *V. vinifera* cv Cabernet Sauvignon scions grown on 18 different rootstocks, namely: Riparia Gloire (*V. riparia*); *V. rupestris* cv St. George; Salt Creek (*V. champinii*); Couderc 3309 (3309C), Milardet et de Grasset 101-14 (101-14 Mgt), Schwartzman (all *V. riparia* × *V. rupestris*); Richter 110 (110 R), Ruggeri 140 (140 Ru), Paulsen 1103 (1103 P), Oppenheim #4 (SO4), Kober 5BB (5BB), Millardet et de Grasset 420A (420A), and Teleki 5C (5C), (all *V. berlandieri* × *V. riparia*); Couderc 1616 (1616C) (*V. solonis* × *V. riparia*); Harmony, Freedom (both open pollination seedlings of 1613C × Dog Ridge); 039-16 (*V. vinifera* × *Muscadinia rotundifolia*); and Boerner (*V. riparia* × *V. cinerea*). The GLRaV-2 type was selected based on producing characteristic leafroll disease symptoms on Cabernet Sauvignon. The source plant also tested positive by ELISA and RT-PCR for GLRaV-2. Chip-bud inoculated plants from a source that tested negative for a full panel of RT-PCR for viruses associated with leafroll disease, including GLRaV-2RG and *Grapevine rupestris stem pitting associated* virus (GRSPaV) as well as plants without chip-bud inoculation were used as controls.

Two test plants of each rootstock were chip-bud inoculated (three buds per plant) onto the scion portion and the viability of inoculum bud chips was visually assessed 30 days post-inoculation. Test plants were established and maintained in the field and results were obtained at the end of a 2-year incubation period. In the next growing season following graft inoculation, test plants were inspected for foliar symptoms. At the end of the 2-years incubation period, the surviving inoculated plants and healthy controls were sacrificed and the woody cylinders examined for wood markings. The same sources of Redglobe and GLRaV-2 type grapevines were used to graft-inoculate *V. rupestris* cv. St. George plants, an indicator host for GRSPaV.

In addition, bioassays through sap transmission of GLRaV-2RG and -2 type strain were compared on the herbaceous plants: *Chenopodium quinoa*; *C. amaranticolor*; *Nicotiana benthamiana*; *Gomphrena globosa*; *N. occidentalis;* and *N. clevelandii*. Young grape leaves or, alternatively, in vitro–grown young shoots (1.5-months-old) from infected Redglobe and GLRaV-2 type sources were ground in a mortar and pestle with cold 0.05 M (Na/K) phosphate buffer pH 7.2, containing 2.5% nicotine [29]. The inoculum was rubbed onto celite-dusted leaves of herbaceous plants, which were maintained in a glasshouse at 24°C and 16-h light period for 4 weeks and visually inspected for symptoms.

Genome-sequencing and sequence analysis

Viral dsRNAs were purified from cambial scrapings of dormant canes, digested with nucleases DNase and RNase (RNaseA was used with NaCl concentration of 0.3 M) to eliminate DNA and single-stranded RNA contaminants [30, 31]. Double-stranded RNAs were then used, after heat denaturation, for cDNA synthesis and cloning using Superscript II cDNA synthesis kit (Invitrogen Inc.,

Carlsbad, CA) following manufacturer's procedures. *Taq* DNA polymerase was used to the 3′ ends of cDNAs proceeding ligation to the pCRII-TOPO TA vector (Invitrogen, Carlsbad, CA) and transformation to *E. coli* cells by electroporation [32]. Gaps between sequences were bridged using specific primers flanking the gap region. Two-step reverse transcriptase polymerase chain reaction (RT-PCR) was done using SuperScript II Reverse Transcriptase, *Taq* DNA polymerase and dsRNA as a template. The fragments were cloned and sequenced (at least five clones from each gap region were sequenced and checked for identity). The 5′-end sequence was determined using the Smart PCR cDNA library constructing kit (Clontech Laboratories, Inc., Palo Alto, CA) in combination with viral-specific primers. To sequence the 3′-end, the dsRNAs were polyadenylated using the yeast poly(A) polymerase (USB Corp. Cleveland, OH) at 30°C for 30 min and the tailed dsRNA was used as template in a reverse-transcription reaction. Complementary DNA clones were sequenced using the ABI Prism 377 DNA sequencer (Perkin Elmer, Foster City, CA) at the University of California, Davis facility, using both universal and virus-specific primers. Multiple overlapping clones were sequenced from both directions. Sequences were analyzed using Wisconsin GCG software package (Genetic Computer Group, Madison, WI) and Web-based database searching and analysis found in NCBI. Identification of major open-reading frames (ORFs), translated protein sequences, and conserved domains were done with ORF finder, BLASTN, and BLASTX (National Center for Biotechnology Information [NCBI]; http://www.ncbi.nlm.nih.gov). Alignment analysis was done with ClustalW (European Bioinformatics Institute [EBI]; http://www.ebi.ac.uk). Amino acids composition of encoded proteins and putative molecular weights were determined using BioEdit Sequence Alignment Editor Program [33].

Sequences of the complete HSP70h gene of GLRaV-2RG and 32 other members within the family *Closteroviridae* were used in a phylogenetic analysis. Partial sequences of this gene were available for GLRaVs-4, -5, and -7. Beside GLRaV-2RG that deposited in the Genbank under the Grapevine rootstock stem lesion associated virus (GRSLaV; NC_004724), the following viruses were used in this comparison included: Beet yellows virus (BYV; NC_001598); Citrus tristeza virus (CTV;NC_001661); Sweet potato chlorotic stunt virus (SPCSV; NC_004124); Cucurbit yellow stunting disorder virus (CYSDV; NC_004810); Beet pseudo-yellows virus (BPYV; NC_005210); Strawberry pallidosis-associated virus (SPaV; NC_005896); Mint virus -1 (MV-1; NC_006944); Blackberry yellow vein associated virus (BYVaV; NC_00696); Tomato chlorosis virus (ToCV; NC_007341); Strawberry chlorotic fleck associated virus (SCFaV; NC_008366); Plum bark necrosis-stem pitting associated virus (PBNSPaV; NC_009992); Grapevine leafroll associated virus-10 (GLRaV-Pr; NC_011702); Carrot yellow leaf virus (CYLV; NC_013007); Tomato infectious chlorosis virus (TICV; NC_013259); and Lettuce infectious yellows virus (LIYV; NC-003618). Other closteroviruses isolated from grapevines with leafroll disease symptoms included: Grapevine leafroll associated viruses*:* GLRaV-1 (AF195822); GLRaV-2 (AF039204); GLRaV-3 (AF037268); GLRaV-4 (AF039553); GLRaV-5 (AF039552); GLRaV-7 (Y15987); GLRaV-9 (AY297819); GLRaV-11 (GLRaV-De; AM494935) and Grapevine leafroll associated carnelian virus (GLRaCV; FJ907331). In addition, sequences of other members of the family *Closteroviridae* were used in the comparative study and phylogenetic analysis, such as Pineapple mealybug wilt associated viruses*:* PMWaV-1 (AF414119); PMWaV-2 (AF283103) and PMWaV-3 (DQ399259). Also Potato yellow vein virus (PYVV; AF150984); Carnation necrotic fleck virus (CNFV; EU884443); Little cherry virus-1 (LChV-1; Y10237); and Beet yellow stunt virus (BYSV; U51931) were included. Dacus carota (DC; X60088) was used as an outgroup. The sequences were multialigned by using the ClustalW program and a phylogenetic tree was constructed using MEGA5 [34]. Bootstrap values (1,000 replicates) were shown next to the branches [35]. Evolutionary history was inferred using the Neighbor-Joining method of Saitou and Nei [36]. Evolutionary distances were computed using the Maximum Composite Likelihood method [37] and expressed as the number of base substitutions per site.

Antisera production and serological testing

Recombinant viral protein was produced by fusing the gene encoding GLRaV-2RG coat protein into the pQE30 vector (Qiagen, Germany), in the presence of a 6xHis-tail at the N-terminus to allow purification with nickel-nitriloacetic acid (Ni-NTA) metal affinity chromatography [38]. Following transformation of *E. coli* strain M15, protein expressed by the vector was accumulated as insoluble inclusion bodies, and hence required purification [39]. For polyclonal antibody production, purified recombinant coat protein (about 200 μg per injection) was injected into a rabbit over three weekly intervals according to the procedure described by Abou-Ghanem et al. [40].

Cell lines of monoclonal antibodies were produced by Dr. Donato Boscia (IVV-CNR, Bari, Italy) to GLRaV-2 isolate H4 purified from *N. benthamiana*. These cell lines and the polyclonal antibodies produced for GLRaV-2RG (in the preceding) were tested in TAS-ELISA in different combinations as capture and decoration antibodies [41]. Western blot analyses were carried out using purified recombinant coat protein and extracts of grapevine cambial

scrapings described by Uyemoto et al. [42]. The proteins were transferred to PVDF membrane (Immobilon, Millipore) and incubated at room temperature for 1 h with the specific recombinant antiserum. Membranes were washed with TBS-T buffer and stained using the Immuno-Blot kit following the manufacturer protocol (Bio-Rad). Another source of antibodies used in Western blot analysis was a polyclonal antiserum specific to GLRaV-2, a gift from Dr. Dennis Gonsalves (Cornell University, Department of Plant Pathology; New York State Agricultural Experiment Station, Geneva, NY 14456).

Virus detection in test plants of Cabernet Sauvignon and commercial vineyard collections

Presence of GLRaV-2RG in graft-inoculated asymptomatic test plants of Cabernet Sauvignon on 13 rootstocks and a collection of 920 grapevines in California commercial vineyards, which included 384 varieties and clones, were analyzed by one-step RT-PCR assay [43]. The primers used were designed within the GLRaV-2RG HSP70h region. The primers (RGHSP227 forward primer, 5′-GCGACTCCAG CAACTTTAGTGA-3′ and RGHSP777 reverse primer, 5′-GTCTAACGAAAGATCGGGTTCTAAG-3′) were tested on a panel of known infected grapevines with GLRaV-1, to -7, and -9, GRSPaV, *Grapevine virus*—A, -B, and -D (GVA, GVB, and GVD), *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), and *Tomato ringspot virus* (ToRSV). The primer set for GLRaV-2RG amplified a product of 551 bp.

Results

Biological assessment

Chip buds of sources Redglobe and GLRaV-2 type grapevines were inserted onto *V. rupestris,* cv. St. George (indicator host for GRSPaV) developed pit symptoms on the woody cylinders. Also, Redglobe inoculum grafted onto test plants of Cabernet Sauvignon scions on five rootstocks (1103P, 1616C, 3309C, 5BB, and 5C) developed poorly and a few plants succumbed within 2 years in the field. At 1 year post inoculation, canopies of solid red coloration (Fig. 1a) developed on test plants grown on these five rootstocks. Two years post-inoculation, symptomatic surviving test plants were nurtured to fall season, sacrificed, trunk sections autoclaved briefly, bark stripped, and examined for wood markings. On diseased, but not healthy grapevines, woody cylinders exhibited necrotic lesions, such as shown on rootstock 1616C (Fig. 1b). In the trial, GLRaV-2RG infections on all remaining test plants (i.e. on the remaining 13 rootstocks) were asymptomatic. Co-infection status by GRSPaV was confirmed in the GLRaV-2 type and Redglobe sources by RT-PCR (see below). Typical symptoms of leafroll disease only were incited by GLRaV-2 type strain across test plants on all 18 rootstocks. However, Cabernet Sauvignon test plants inoculated with both virus sources, but not control plants, developed stem pitting on the St. George under-stocks characteristic of GRSPaV. All GLRaV-2RG herbaceous plant bioassays were negative. In contrast, GLRaV-2 type strain was successfully transmitted to *N. benthamiana*.

Complete genomic sequence and comparative analysis

A high molecular weight dsRNA band, typical for closteroviruses, was purified from Redglobe bark tissues and complementary DNAs produced from them. The cDNA was cloned into plasmid TOPO TA, and 50 clones were sequenced. Gaps in the sequence data were bridged using specific primers to generate overlapping coverage over the viral genome, using dsRNA as template. The complete genome of GLRaV-2RG totaled 16,527 nts (submitted to GenBank under accession number [AF314061 under GRSLaV] and Refseq [NC_004724]). The genome encoded nine open-reading frames (ORFs) (Fig. 2). ORF1a encodes a polyprotein (327.4 kDa product size) with PRO, MTR, and HEL domains. Two sections of the papain-like protease (P-PRO) domain were identified by amino acid homology, sharing 39% similarity to each other. The P-PRO domains were located in the 5′-region of ORF1a. The putative catalytic cysteine and histidine residues were predicted at positions 492: 552 and 761: 820, respectively. Presumed cleavage sites of P-PRO were between amino acid residues Gly-Gly (571-572) and Ala-Gly (840-841). The *N*-terminal fragment of the ORF1a product consisted of the 2,091 amino acids for the MTR and HEL domain. This region was found to share 77% amino acid sequence identity with the putative MTR-HEL domain of GLRaV-2 type strain, the closest known relative as revealed by a BLAST-P search. Both viruses carry conserved motifs typical of positive-stranded viral RNA type I MTRs [44]. ORF1b appears to be translated through ribosomal frameshift [45–48] and encodes a polypeptide of 459 amino acids long (counting from the putative frameshift site) and predicted molecular weight of $\sim$52.6 kDa. ORF1b contains eight conserved motifs characteristic of viral RNA-dependent RNA polymerase [44]. The function of ORF2, which encodes a polypeptide of 57 amino acids with a molecular weight of 6.4 kDa, is not known yet. ORF3 translates to a 599 amino acids polypeptide of molecular weight 65.4 kDa with homology to the heat shock 70 protein-homologue (HSP70h) sequences and carries eight motifs with homology to those of the HSP70h gene of aphid-borne closteroviruses. ORF4 translates to a 534 amino acid polypeptide

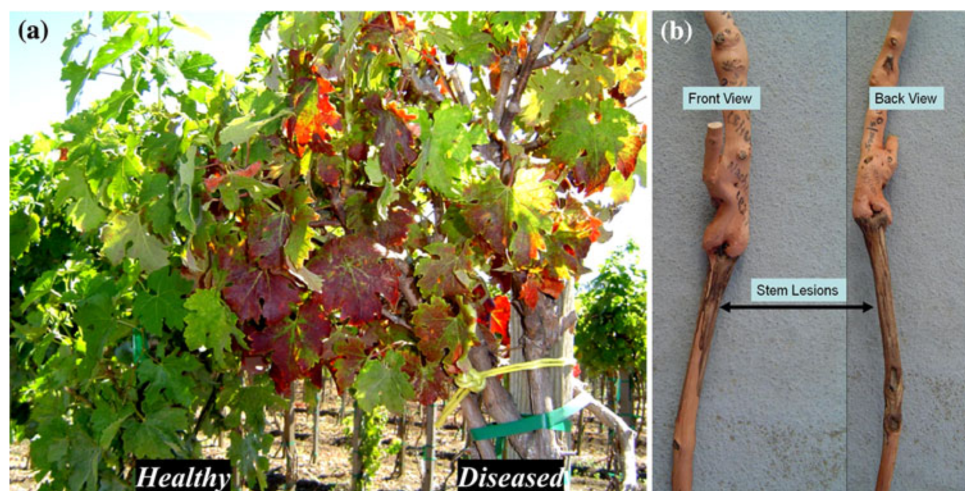

Fig. 1 Symptom expression of Cabernet Sauvignon on rootstock 1616C graft-inoculated from a Redglobe grapevine infected with GLRaV-2RG. **a** Red leaf symptoms 1 year post graft inoculation. **b** Stem lesion on the woody cylinder of the rootstock 1616C; 2 years post inoculation

of molecular weight 61.8 kDa with homology to the heat shock 90 protein (HSP90) sequences. This predicted protein has 17 amino acid residues less than GLRaV-2 type at the *C*-terminus, but is almost the same size as the corresponding proteins of BYV and BYSV. ORFs 5 and 6 were identified as genes for the minor coat protein (CPm, molecular weight 24.7 kDa) and major coat protein (CP; molecular eight 21.6 kDa), respectively. The deduced amino acid sequence of ORF6 showed similarities to CPs of GLRaV-2 type, BYV, BYSV, MV-1, CYLV, RpLMV, CTV, and SCFaV. Among these, ORFs 5 and 6 showed highest similarity to GLRaV-2 type CP (90%); and CPm (89%). The amino acids residues, R and D, which are conserved in the CPs of all the filamentous plant viruses and their putative functions are to stabilize CP core region [49], were also identified. ORFs 7 and 8, common among members of the genus *Closterovirus*, were designated based on predicted product sizes of 19 and 24 kDa and composed of 161 and 205 amino acid residues, respectively. p24 showed closest similarity with GLRaV-2BD strain with 85% identity by a BLAST-P search, which has a putative silencing suppressor for the virus [50]. The 3′-UTR of GLRaV-2RG consisted of 223 nucleotides with an extended oligo(A) tract similar to that found with GLRaV-2 type, BYSV, and BYV [51, 52]. The RNA of this virus terminated (at 3′ end) with GCU distinct from that of GLRaV-2 type, BYV, BYSV, or CTV.

Antibody production and serological characterization

Polyclonal antiserum produced against viral coat protein expressed from recombinant protein in *E. coli* reacted with extracts from infected tissues of GLRaV-2RG and GLRaV-2 type strain, but not with healthy tissues (Fig. 3a). Similar results were obtained when using GLRaV-2 type strain polyclonal antiserum against GLRaV-2RG coat protein in ELISA test. This positive serological cross-reactivity was further confirmed using monoclonal antibodies from 18 different cell lines derived against purified virus particles of the GLRaV-2 isolate H4 and the results were similar as described by Zhou et al. [41]. In Western blot analysis, Redglobe tissue extracts reacted weakly against an antiserum of GLRaV-2 produced by Dr. Dennis Gonsalves (Fig. 3b). However, using TAS-ELISA, Redglobe tissue extracts produced OD values ten times more than healthy controls after 30-min incubation.

RT-PCR assays of Cabernet Sauvignon test plants and commercial vineyard collections

RT-PCR assays of the Redglobe grapevine used as the inoculum source, and asymptomatic test plants of Cabernet Sauvignon tested positive for both GLRaV-2RG and GRSPaV. Among the 920 grapevine collection, RT-PCR assays revealed 1.7% incidence of GLRaV-2RG and 13% incidence of GLRaV-2 type in these samples. Mixed infections are relatively common for many viruses affecting vegetatively propagated perennial crop plants and expected. However, single collections of only two grapevine varieties, *Pribidrag* and *Chardonnay*, assayed positive for both GLRaV-2 type and -2RG.

Discussion

With the inclusion of GLRaV-Pr, GLRaV-De, and GLRaV-Carn [8, 9], there are currently 11 recognized viral species associated with leafroll disease (symptoms composed of green primary leaf veins against a background of red tissues and downward rolling in the red-fruited grapevines, such as *V. vinifera* cv, Cabernet Sauvignon, or Cabernet Franc). Ten GLRaVs are members (or putative

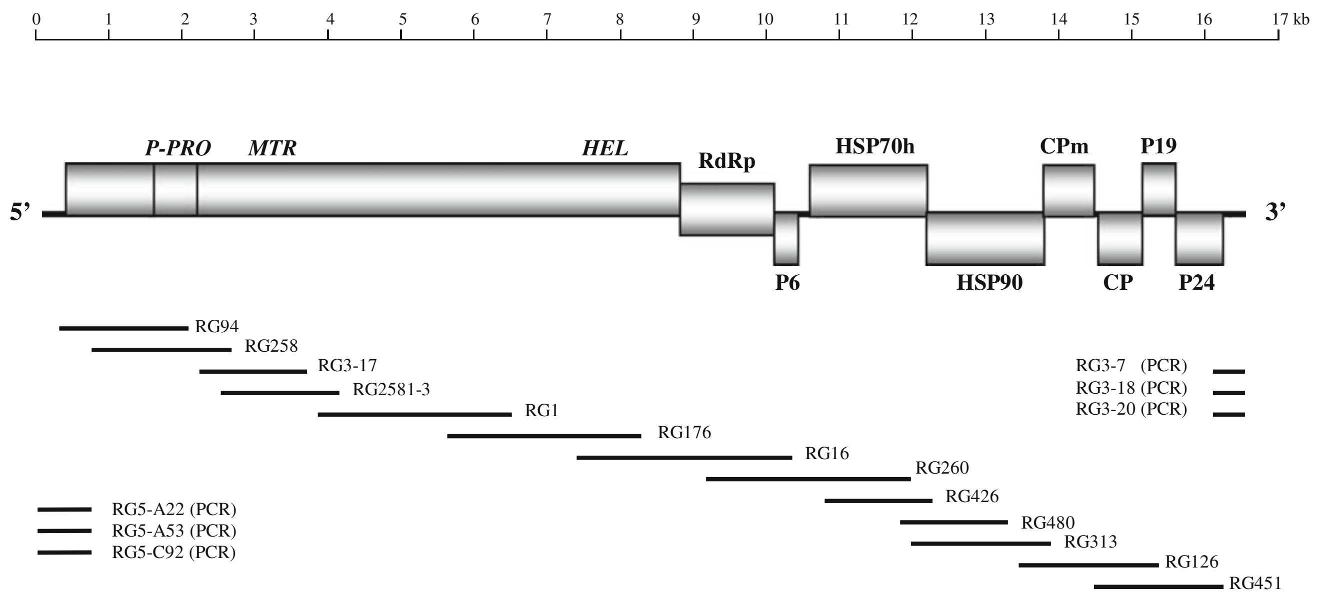

Fig. 2 Schematic representation of the virus genome organization showing the relative position of predicted ORFs illustrated as follows: ORF 1a protein domains for papain-like protease (PRO), methyltransferase (MTR), and helicase (HEL); ORF 1b, the putative virus RNA-dependent RNA polymerase (RdRp); ORF 2, small size protein P6; ORF 3, homologue to cellular heat shock related protein 70 (HSP70h); ORF 4, homologue to heat shock related protein 90 (HSP90h); ORF 5, the putative minor coat protein (CPm); ORF 6, the virus major coat protein (CP); ORF 7 and ORF 8, consisting the proteins P19 and P24, respectively. *Bars* represent overlapping clones obtained from the cDNA library. The 5′ and 3′ end were correctly identified by sequencing several clones at each terminus

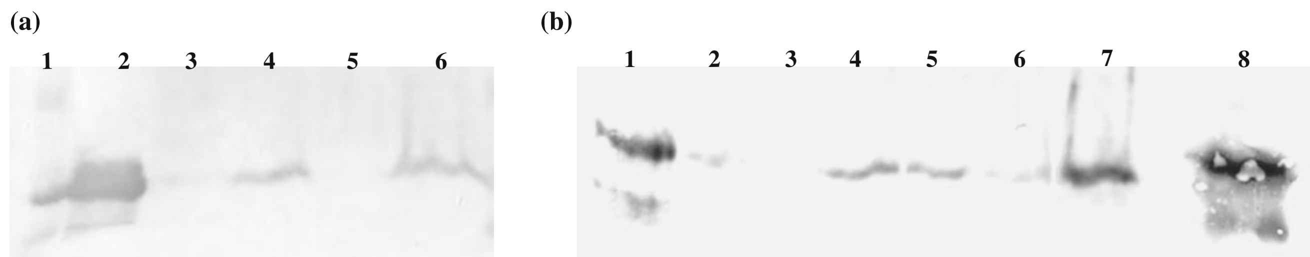

Fig. 3 **a** Western blot analysis using antiserum produced to recombinant GLRaV-2RG CP. Samples included: (1) prestained protein marker (NEB P7708S); (2) *N. benthamiana* infected by GLRaV-2 type; (3) healthy grapevine, cv Primitivo; (4) GLRaV-2 type-infected grapevine cv Primitivo; (5) healthy grapevine, cv Redglobe; (6) GLRaV-2RG-infected Redglobe grapevine. **b** Western Blot analysis of Redglobe infected grapevine extracts (*lanes* 1–8) as a source of antigens against polyclonal antisera of GLRaV-2 (Courtesy of D. Gonsalves)

members) of the genus *Ampelovirus*. The only exception is GLRaV-2 in the genus *Closterovirus* [53]. However, whereas type strain of GLRaV-2 caused leafroll symptoms, GLRaV-2RG infections were latent in own-rooted grapevines of Redglobe, Cabernet Sauvignon, and Chardonnay (Uyemoto unpublished data). Discovery of GLRaV-2RG was made by graft-inoculations of Redglobe bud chips onto test plants of Cabernet Sauvignon on five rootstocks, namely, 3309C, 1103P, 1616C, 5C, and 5BB. The uniform red colored canopies observed on infected test plants of Cabernet Sauvignon scions propagated on these sensitive rootstocks were a consequence of a hypersensitive (necrotic) response to the invasion of GLRaV-2RG (Fig. 1a); an unprecedented symptomatic anomaly among closterovirus infections. GLRaV-2RG also caused latent infections in test plants of Cabernet Sauvignon on another 13 rootstocks [54]. In contrast, infections by GLRaV-2 type caused only leafroll symptoms and no woody cylinder markings. Several isolates of GLRaV-2 were mechanically transmissible to the herbaceous host *N. benthamiana* [17, 18, 40], but GLRaV-2RG was not.

A high-molecular weight dsRNA band was present in extracts of virus-infected Redglobe grapevines [44] and subsequent molecular analysis revealed a viral genome length of 16,527 nts with nine ORFs identical to genome organization found in species member of the family *Closterovirdae* and genus *Closterovirus*, designated as GLRaV-2RG. Its genome was compared with other GLRaV-2

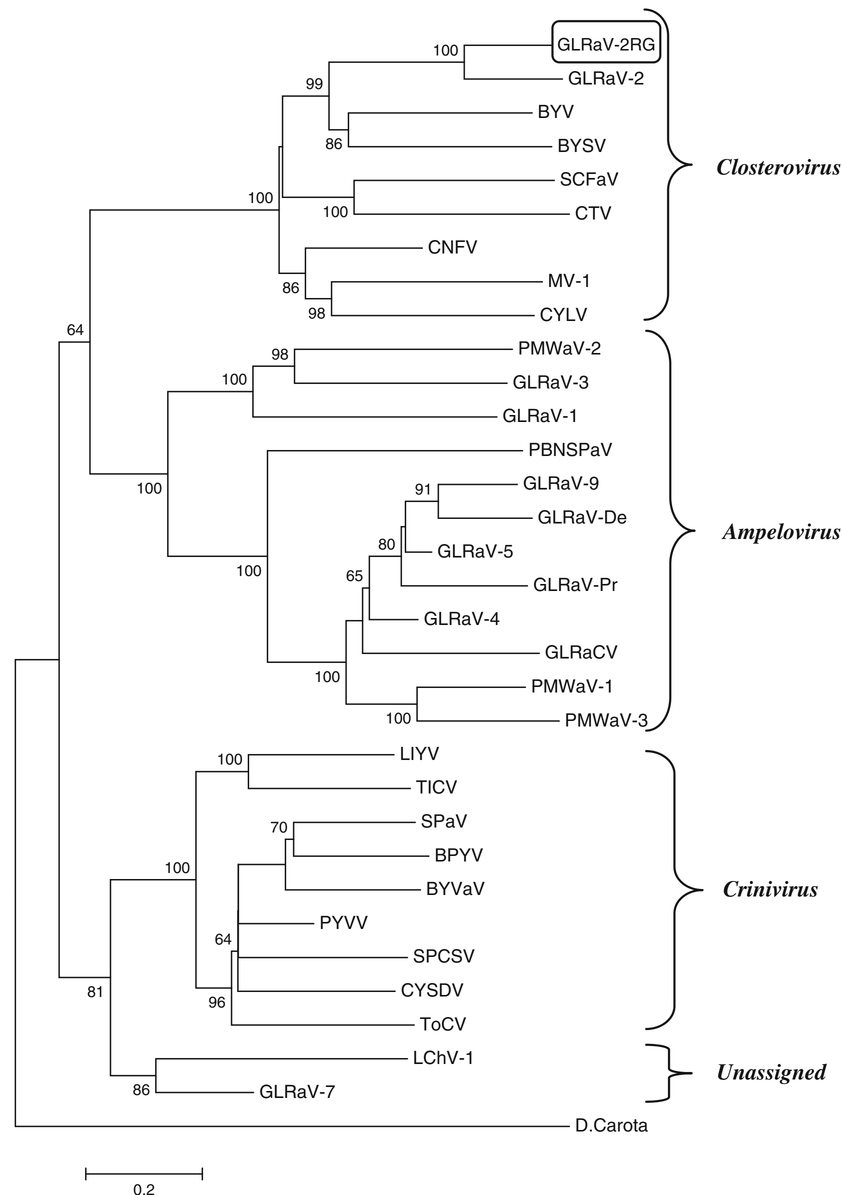

Fig. 4 Phylogenetic analysis showing the relationships of GLRaV-2RG to members of the family C*losteroviridae* based on the nucleic acid sequences of HSP70h gene. GLRaV-2RG found to cluster within members in the Genus *Closterovirus*. Sequences were multialigned using ClustalW program and MEGA5 used for Phylogenetic tree construction. The evolutionary history was inferred using the Neighbor-Joining method while the evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees (>60%) in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The differences in the composition bias among sequences were considered in evolutionary comparisons [70]. The analysis involved 33 nucleotide sequences from the following sources: GLRaV-2RG (NC_004724); BYV (NC_-001598); CTV (NC_001661); SPCSV; (NC_004124); CYSDV (NC_004810); BPYV (NC_005210); SPaV (NC_005896); MV-1 (NC_006944); BYVaV (NC_00696); ToCV (NC_007341); SCFaV (NC_008366); PBNSPaV (NC_009992); GLRaV-Pr (NC_011702); CYLV (NC_013007); TICV (NC_013259); LIYV (NC-003618); GLRaV-1 (AF195822); GLRaV-2 (AF039204); GLRaV-3 (AF037268); GLRaV-4 (AF039553); GLRaV-5 (AF039552); GLRaV-7 (Y15987); GLRaV-9 (AY297819); GLRaV-De (AM494935); GLRaCV (FJ907331); PMWaV-1 (AF414119); PMWaV-2 (AF283103); PMWaV-3 (DQ399259); PYVV (AF150984); CNFV (EU884443); LChV-1 (Y10237); BYSV (U51931), and (DC; X60088) as outgroup

sequences, e.g., isolates BD from Italy (DQ286725), 93/955 from South Africa (NC_007448), and OR1 from Oregon (FJ436234). Isolate OR1 showed 99% identity with GLRaV-2 type and 73% identity with GLRaV-2RG. Genome length of GLRaV-2RG was 41 nts longer than isolate OR1, and 8 nts shorter than isolate BD, which shared 79%

Table 1 Comparison of nucleotide (nt) and amino acid (aa) sequences (percent identity) of corresponding genes of grapevine leafroll associated virus-2 type; BD and 93/955 with -2RG

GLRaV-2 isolate		Overall	5′NCR	ORF1a	RdRp	P6	HSP70 h	HSP90	CPm	CP	P19	P24	3′UTR
Type strain	*na*	–	–	71	79	71	73	73	78	76	76	77	77
(AF039204)	*aa*	–	–	75	94	73	82	79	89	90	77	80	
BD	*na*	79	92	78	81	82	81	79	81	79	83	85	86
(DQ286725)	*aa*	–	–	82	96	86	92	87	92	86	83	84	
93/955	*na*	73	86	71	79	74	74	78	77	76	77	76	67
(NC_007448)	*aa*	–	–	72	93	72	83	80	89	90	78	78	

identity in total nucleic acid sequences. Comparisons between GLRaV-2RG and isolate 93/955 showed 73% identity and the latter had 49 nts less.

Based on nucleotide sequences for the HSP70h, a phylogenetic tree was generated [15, 55–59]. The phylogram obtained showed GLRaV-2RG clustered with members in the genus *Closterovirus*; closely related to GLRaV-2 type strain and distantly to BYV and BYSV (Fig. 4). Using the Web-based bioinformatics program, nine ORFs were identified and compared with GLRaV-2 type strain, the closest identified member species in the genus *Closterovirus*. The genome of GLRaV-2RG revealed presence of two putative P-PRO domains at the *N*-terminus of ORF1a and a predicted corresponding autoproteolytic cleavage site. Conserved motifs of the replication-associated proteins were identified and found to be similar to other closteroviruses, most notable GLRaV-2 type at the RdRp region (94% identity). Complete sequence comparisons of GLRaV-2 type and GLRaV-2RG demonstrated reading-frame similarities ranging from 71 to 79% at the nucleotide level and 73 to 94% at the amino acid level (Table 1). Amino acid sequences showed similarities of 89 and 90%, respectively, for CPm and CP.

The identity between GLRaV-2RG and GLRaV-2 type, suggests that GLRaV-2RG is a strain of GLRaV-2 [60, 61]. Other strains of GLRaV-2 have been reported in New Zealand “Alfie virus” [62] and Chile [63]. In Australia, a strain of GLRaV-2 was found to be associated with young vine decline inducing graft incompatibility and possibly different from GLRaV-2RG [1]. Other virus isolates denoted “PN” (from Pinot Noir), “93/955”, “H4”; “BD”; Sem and OR1 also have been identified and proposed as strains of GLRaV-2 [14, 15, 64–66]. Comparison of corresponding genes of GLRaV-2RG with isolates BD and 93/955 (Table 1), revealed closest amino acid identity of BD strain to RG (82-96%) in all ORFs except for CP (86%), whereas with GLRaV-2 type CP was 90%. GLRaV-2RG clustered with GLRaV-2BD, which also induced graft incompatibility and failure to induce leafroll symptoms [67, 68]. Among 920 grapevine collections from commercial vineyards in California, 1.7% tested positive for GLRaV-2RG and 13.5% for GLRaV-2 by RT-PCR assays. Only two plants were co-infected with both GLRaV-2 and –2RG.

Serological correlation was used to separate GLRaV-2 type from GLRaV-6 [25, 69], and GLRaV-5 from GLRaV-9 [7]. However, GLRaV-2 type and -2RG proved to be serologically related even though they are biologically distinct [28]. Furthermore, the sequencing information reported here also supports the serological data suggesting that these two viruses are closely related. Therefore, we suggest Grapevine leafroll associated virus-2 Redglobe strain (GLRaV-2RG) as an official name for this virus.

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