

Biological, Serological, and Molecular Characterization of a Highly Divergent Strain of *Grapevine leafroll-associated virus 4* Causing Grapevine Leafroll Disease

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ABSTRACT

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The complete genome sequence of a highly divergent strain of *Grapevine leafroll-associated virus 4* (GLRaV-4) was determined using 454 pyrosequencing technology. This virus, designated GLRaV-4 Ob, was detected in *Vitis vinifera* ‘Otcha bala’ from our grapevine virus collection at Agroscope. The GLRaV-4 Ob genome length and organization share similarities with members of subgroup II in the genus *Ampelovirus* (family *Closteroviridae*). Otcha bala was graft-inoculated

onto indicator plants of cultivar Gamay to evaluate the biological properties of this new strain, and typical leafroll symptoms were induced. A monoclonal antibody for the rapid detection of GLRaV-4 Ob by enzyme-linked immunosorbent assay is available, thus facilitating large-scale diagnostics of this virus. Based on the relatively small size of the coat protein, the reduced amino acid identity and the distinct serological properties, our study clearly shows that GLRaV-4 Ob is a divergent strain of GLRaV-4. Furthermore, molecular and serological data revealed that the AA42 accession from which GLRaV-7 was originally reported is in fact co-infected with GLRaV-4 Ob and GLRaV-7. This finding challenges the idea that GLRaV-7 is a leafroll-causing agent.

Similar to other woody perennial crops, grapevines (*Vitis* spp.) are prone to infection by diverse viruses. Currently, more than 60 viruses have been reported to infect grapevines (Martelli 2014). Grapevine leafroll disease (GLRD) is one of the most economically important viral diseases of grapevines, and its effects on yield and harvest quality have been documented for several grapevine cultivars (Komar et al. 2010; Lee and Martin 2009; Mannini et al. 2012; Spring et al. 2012). Cultivars infected with GLRD generally exhibit yield reduction and poor fruit quality. For red grape cultivars, one of the primary effects of GLRD is lower anthocyanin accumulation, thus resulting in poor berry color development. For white cultivars, GLRD symptoms are visually less evident; however, infected grapevines may show chlorotic mottling of leaves toward the end of the growing season.

GLRD has a complex etiology associated with different filamentous viruses referred to as *Grapevine leafroll-associated viruses* (GLRaVs). All GLRaVs identified to date belong to the family *Closteroviridae*. In total, five different GLRaV species have been identified: one in the genus *Closterovirus* (GLRaV-2), three in the genus *Ampelovirus* (GLRaV-1, GLRaV-3, and GLRaV-4), and one in the recently defined genus *Velarivirus* (GLRaV-7) (Al Rwahnih et al. 2012). The genus *Ampelovirus* is further divided into subgroup I, consisting of GLRaV-1 and GLRaV-3 and subgroup II, consisting of all the genetically divergent GLRaV-4 strains (Abou Ghanem-Sabanadzovic et al. 2012). According to the most recent taxonomic

revision of the genus *Ampelovirus*, GLRaV-5, GLRaV-6, GLRaV-9, GLRaV-Pr, and GLRaV-Car were classified as strains of GLRaV-4 and not, as had been previously assumed, as distinct species in the genus *Ampelovirus* (Abou Ghanem-Sabanadzovic et al. 2010, 2012; Maliogka et al. 2009; Martelli et al. 2012).

Herein, we report the description of a filamentous virus infecting a grapevine accession and showing leafroll symptoms when grafted onto cultivar Gamay indicators. We present its complete genome sequence, describe the genome organization and serological features, and show that this virus is a highly divergent strain of GLRaV-4. Finally, using a combination of serological and molecular diagnostic techniques, we show that accession AA42 is co-infected with GLRaV-7 and GLRaV-4 Ob. The implication of these findings for leafroll etiology is discussed.

MATERIALS AND METHODS

Virus isolates and biological indexing. The primary grapevine materials used for this study were collected from the grapevine virus collection at Agroscope in Nyon (Switzerland), which contains more than 600 clones of distinct plant accessions (Gugerli et al. 2009a). Three cuttings from the Otcha bala grapevine accession (numbers 10,496, 10,497, and 10,498) were used for biological, serological and molecular characterization. Additional grapevine accessions used for this study included AA42, Y276, and Chiliaki Chjornyj, which were kindly provided by W. Jelkmann, O. Lemaire, and the National Institute of Agrobiological Sciences (Japan), respectively. The accession Chiliaki Chjornyj was shown to be co-infected by GLRaV-7 and GLRaV-4 strain Ru (Ito et al. 2013). Three additional GLRaV-7-infected accessions were provided by A. Rowhani from UC Davis: Siar, Takhani, and Sultanina rose. Using microsatellite analysis, the cultivar identity of the Otcha bala plant accession was verified, and grapevine accession AA42 was identified as the grapevine cultivar Sultanine (E. Droz, *personal communication*).

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Otcha bala canes were graft-inoculated onto the leafroll-specific indicator *Vitis vinifera* 'Gamay Rouge de la Loire'. Eight replicates were planted in the field, and symptoms were evaluated over a 3-year period. Graft-inoculated GLRaV-1-infected vines were grown as positive controls.

Virus particle purification and serology. Virus particles were purified from mature leaves as described previously (Gugerli et al. 1984). Purified virus particles were observed using a Philips CM10 transmission electron microscope, as described by Gugerli and Ramel (2004).

A cell line producing the monoclonal antibody MAb37a was generated against viruses purified from accession Y276 (Rigotti et al. 2006). The serological tests used in this study consisted of double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs), immunoprecipitation electron microscopy (IPEM), and western blot. These tests were performed essentially as described previously (Gugerli and Ramel 2004).

Commercially available ELISA kits (GLRaV-1 DAS, GLRaV-2 DAS, GLRaV-3 DAS, and GLRaV-6 DAS from Bioreba AG, Switzerland) were used to screen for the indicated grapevine leafroll-associated viruses according to the manufacturer's instructions. DAS-ELISAs using reference antisera and monoclonal antibodies developed at Agroscope were used to test for GLRaV-4 infection. Briefly, ELISA plates were first coated with rabbit antiserum (1 µg/ml) in carbonate buffer and then incubated with grapevine crude leaf extracts for 16 h at 6°C. Then, the wells were washed, and alkaline phosphatase-conjugated monoclonal antibodies were added. To detect GLRaV-4, GLRaV-4 strain 5, and GLRaV-4 strain 9, the following monoclonal antibodies were used: MAb 3-1, MAb 3-3, and MAb 27-1, respectively (Besse et al. 2009; Gugerli et al. 2009b). The reaction with the chromogenic substrate p-nitrophenyl phosphate was performed at room temperature, and the absorbance at 405 nm was read using a spectrophotometer after 3 h.

Nucleic acid extraction, RT-PCR amplification, and Sanger sequencing. Total RNA was extracted from mature leaf petioles using RNeasy Plant Mini Kits (Qiagen, Germany). One-step reverse transcription-polymerase chain reaction (RT-PCR) was performed using the AMV reverse transcription (Promega, Germany) and GoTaq polymerase (Promega, Germany) with total RNA as the template. RT-PCR was performed with primer pairs specific for each virus using the conditions described in the original publications (Supplementary Table S1).

For sequencing purposes, purified PCR products were cloned into the vector pGEM-T (Promega, Germany) and were sequenced at FASTER SA (Switzerland). To sequence the 3'-end of the GLRaV-4 Ob genome, viral RNAs were polyadenylated using an A-Plus Poly (A) Tailing Kit (Epicentre Biotechnologies, Madison), and the tailed viral RNA was used as the template in a reverse-transcription reaction. Sequences of the 5' and 3' viral termini were obtained using a 5'/3' RACE kit (Roche). Two independent clones were sequenced from each 5' and 3' terminus.

Virus particle enrichment, pyrosequencing, assembly, and sequence analyses. Purified viral particles were treated with nucleases (DNase and RNaseA) to remove *Vitis* DNA and RNA contaminants. Then, viral RNA was extracted from purified viruses using RNeasy Plant Mini kits (Qiagen, Germany) and randomly amplified using a Whole Transcriptome kit (Sigma-Aldrich) for sequencing on a Roche 454 GS Junior platform (Roche Diagnostics Corp., Branford, CT). Sequencing libraries were prepared with a Rapid Library Preparation kit according to the manufacturer's protocol and sequenced on one PicoTiter plate using Titanium chemistry. Quality control analysis and assembly of the produced reads were performed using DNASTAR's NGen assembler (Madison, WI) with 454-specific parameters. Filtered reads were converted to fasta files and subjected to BLASTN analysis (Altschul et al. 1997) with the GenBank nonredundant nucleotide database using decreasing wordsize options of 400, 200, 100, 50, and 28.

Gene annotation was performed following a comparison with sequences from other leafroll-associated viruses and using GeneMarkS software (Besemer and Borodovsky 2005). Amino acid and nucleotide alignments were created using ClustalW (Goujon et al. 2010). The sequences and accession numbers of the viral species/strains used for the amino acid sequence comparisons with GLRaV-4 Ob are provided in Supplementary Table S2. The phylogenetic relationships were determined using Molecular Evolutionary Genetic Analysis software MEGA version 6 with the best amino acid substitution model (Tamura et al. 2013). Phylogenetic trees were generated using the maximum likelihood algorithm with 500 bootstrap replicates.

RESULTS

Electron microscopy and biological indexing. Viral particles were isolated from leaf samples of the Otcha bala accession. Electron micrographs showed filamentous particles consistent with the family *Closteroviridae*, with the most frequent length being 1,600 nm (data not shown). The presence of leafroll disease was assessed by biological indexing onto the leafroll-specific indicator 'Gamay'. Mild leafroll symptoms, including reddening and downcurling of the leaves, were observed during the three consecutive years following the graft inoculation (Fig. 1). Original Otcha bala accessions and graft-inoculated Gamay plants repeatedly tested negative for GLRaV-1, GLRaV-2, GLRaV-3, and GLRaV-4 (and its strains 5, 6, and 9) viruses by ELISA, thus justifying further investigation to characterize the cause of the disease.

Molecular characterization by pyrosequencing. RNA isolated from virus particles purified from the Otcha bala grapevine was submitted to 454 high-throughput sequencing. The analysis yielded 59,087 high-quality reads with an average read length of 430 bp. In total, 13,173 reads were de novo assembled into a 12,882-nt contig with homology to members of the family *Closteroviridae*. The coverage over this contig ranged from 1- to 1,918-fold, as shown in Figure 2.

To verify the results provided by deep sequencing, specific primers were designed using the pyrosequencing data (Supplementary Table S3). Sanger sequencing of PCR products validated the pyrosequencing results. Completion and polishing of the sequence's termini was performed by RACE PCR using Otcha bala cDNA as the template. RACE sequencing of viral termini led to the modification of the 12,882-nt initial contig's extremities, resulting in a complete genome length of 12,849 nt. The genome sequence was deposited in the GenBank database under accession number KP313764.

Virus-derived fragments were identified in the total fragment pool based on their similarities to the nucleotide sequences archived in GenBank using BLASTN. The closterovirus-like virus, which we propose to name GLRaV-4 Ob, was the most prevalent species



Fig. 1. Leafroll symptoms on Gamay graft-inoculated with Otcha bala accession: **A**, downward curling of leaf margins and **B**, interveinal red coloration.

among the 454 data set (Table 1). Three other viruses were also identified in the 454 dataset: two viruses of the family *Tymoviridae* (*Grapevine fleck virus* [GFKV] and *Grapevine red globe virus* [GRGV]) and one member of the family *Betaflexiviridae* (*Grapevine virus A* [GVA]). No other closterovirus-related reads were identified from the 454 run. The presence of these viral species was confirmed by specific RT-PCR analysis or ELISA.

The GLRaV-4 Ob genome is 12,849 nts in length and contains six putative open reading frames (ORFs) (Fig. 2). The GLRaV-4 Ob genome starts with a short 37-nt-long noncoding region. ORF1a encodes a polyprotein (2,076 aa). Different domains were identified in ORF1a, including a methyltransferase (MET, pfam 01660, Pfam database 27.0 [Finn et al. 2014]), AlkB (pfam 03171), and helicase (HEL, pfam 01443). Additionally, ORF1a contains a papain-like protease domain with the catalytic residues Cys²²⁵ and His²⁶⁸ and a predicted cleavage site after Gly²⁸⁵ (Peng et al. 2001).

ORF1a terminates with the sequence auguuUAG (the stop codon of ORF1a is shown in capital letters, while the start codon of ORF1b is underlined); this sequence is presumably involved in a +1 ribosomal frameshift as described for other closteroviruses (Dolja et al. 2006). ORF1b overlaps the last 8 nt of ORF1a and potentially encodes a 526-aa-long protein. ORF1b shows high homology to the RNA-dependent RNA polymerase (RdRp) domain (pfam 00978). The small ORF2 partially overlaps ORF1b by 26 nts and potentially encodes a 46-aa-long hydrophobic protein (p5). ORF3 is situated downstream of p5 after a 144-bp intergenic region and encodes a 533-aa HSP70 homolog (HSP70h) protein similar to other sequenced GLRaV-4s. ORF4 partially overlaps the previous ORF and encodes a 546-aa-long protein homologous to the p60 proteins of other closteroviruses. After a 69-nt-long intergenic region, ORF5 encodes a 261-aa-long protein corresponding to a viral coat protein (CP). The 3'-end proximal ORF (ORF6) encodes a putative p23 protein. ORF6 is in accordance with similarly positioned small peptides encoded by other closteroviruses at the genome's 3' end (Dolja et al. 2006). The genome ends with a 131-nt-long 3' noncoding region.

Serological characterization. The monoclonal antibody MAb37a reacted with Otcha bala grapevine extracts in a DAS-ELISA (Fig. 3). Otcha bala leaf extracts produced optical density (OD) values 30 times higher than healthy controls after 3 h of

incubation. MAb37a was highly specific because it did not react with other GLRaV-4-like viruses (i.e., GLRaV-4, GLRaV-4 strain 5, GLRaV-4 strain 6, and GLRaV-4 strain 9) from infected grapevines in our collection.

MAb37a activity was further assayed by IPFM. The filamentous virions of the Otcha bala grapevine were heavily decorated with MAb37a (Fig. 4). In western blot analysis, MAb37a reacted to a dominant protein with an estimated molecular mass of approximately 33,000 Da (Fig. 5).

RT-PCR assays and a GLRaV-4 Ob survey of the Agroscope grapevine virus collection. A DAS-ELISA assay using MAb37a was used to monitor the prevalence of GLRaV-4 Ob in our grapevine virus collection. An RT-PCR test targeting the HEL domain of ORF1a of GLRaV-4 Ob was developed using GLRaV-4 Ob-F/R primers to confirm the infection status. Three other accessions from our collection tested positive for GLRaV-4 Ob by ELISA and by RT-PCR: Chiliaki Chjornyj, Y276, and AA42 (Fig. 5). To ascertain viral infection, amplicons from these accessions were sequenced, yielding nucleotide sequences with 88 to 96% identity to GLRaV-4 Ob. The three accessions infected with GLRaV-4 Ob were also tested with the primer set LRamp-F/R reported by Abou Ghanem-Sabanadzovic et al. (2012). With this primer set, a fragment with the expected size was amplified from all three accessions (data not shown). Amplicons were sequenced to verify the specificity of PCR products. Amplicon sequence identity varied from 88 to 98%.

Because Ito et al. (2013) reported a mixed infection of GLRaV-4 and GLRaV-7 in a grapevine, we decided to evaluate the GLRaV-7 infection status of the different materials used in this study. GLRaV-7 infection was assessed by RT-PCR using different pairs of specific primers.

TABLE 1. High-throughput sequencing reads for viral species identified from the Otcha bala grapevine using BLASTN analysis

Virus species	Virus family	Total hits
<i>Grapevine leafroll-associated virus 4</i> Ob	<i>Closteroviridae</i>	19,572 reads
<i>Grapevine fleck virus</i>	<i>Tymoviridae</i>	9,002 reads
<i>Grapevine red globe virus</i>	<i>Tymoviridae</i>	2,687 reads
<i>Grapevine virus A</i>	<i>Betaflexiviridae</i>	111 reads

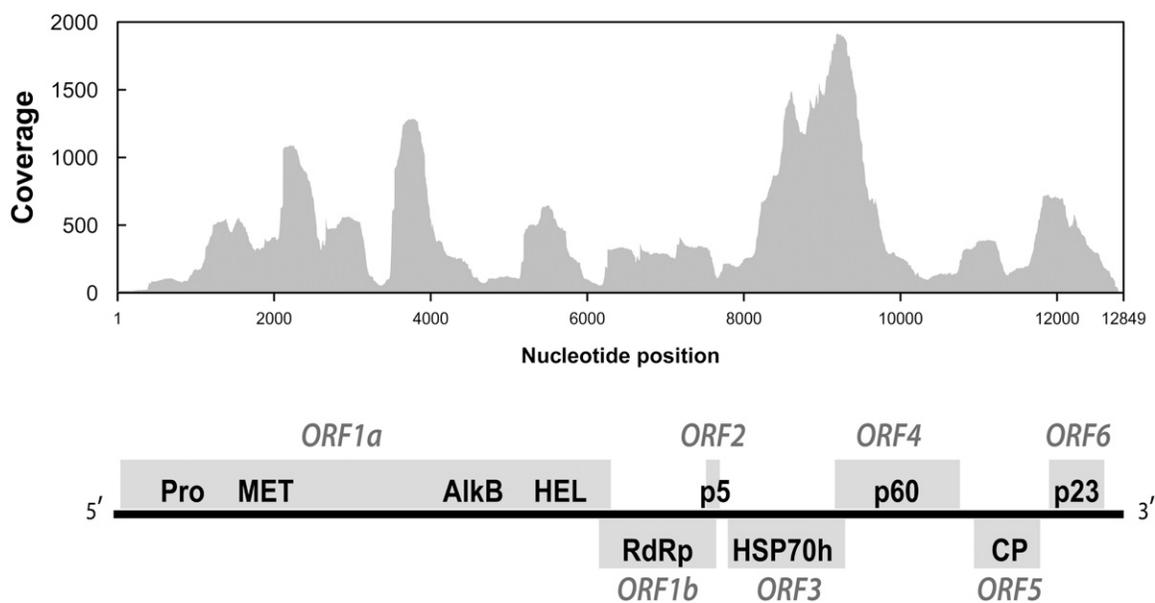


Fig. 2. Sequence coverage and nucleotide positions along the *Grapevine leafroll-associated virus 4* strain Ob (GLRaV-4 Ob) genome. The schematic representation of the GLRaV-4 Ob genome organization is presented to scale. Putative open reading frames (ORFs) are shown in boxes: ORF1a with corresponding domains: Pro = protease, MET = methyltransferase, AlkB = 2OG-Fe(II) oxygenase domain, HEL = helicase; ORF1b = RNA-dependent RNA polymerase; ORF2 = small 5 K protein; ORF3 = heat shock 70 protein homolog; ORF4 = 60 K protein; ORF5 = coat protein; and ORF6 = 23 K protein.

Each RT-PCR amplification product was sequenced to verify its identity. Six accessions tested positive for GLRaV-7 (Fig. 3). The accessions Chiliaki Chjornyj, Y276, and AA42 were co-infected with GLRaV-4 Ob and GLRaV-7. The Otcha bala accession repeatedly tested negative for GLRaV-7 by RT-PCR using five different primer pairs.

DISCUSSION

GLRD has a complex etiology; different viral species belonging to different genera in the family *Closteroviridae* are associated with the disease (Martelli 2014). In this study, we described the infection of an Otcha bala grapevine accession from our viral collection by closterovirus-like virus particles. Graft inoculation of this grapevine accession to the leafroll indicator Gamay resulted in typical leafroll symptoms. To identify the virus responsible for the leafroll symptoms, we characterized the virome of the Otcha bala accession using a pyrosequencing approach. De novo assembly generated a consensus sequence and revealed the presence of a divergent strain of GLRaV-4, which we propose to name GLRaV-4 Ob. Four viruses were identified in the diseased Otcha bala grapevine, including GLRaV-4 Ob in the family *Closteroviridae*. However, GLRaV-4 Ob was the only closterovirus detected in this vine; therefore, this virus was considered to be the agent responsible for the leafroll symptoms observed on the Gamay grapevine.

Similar to other viruses in the family *Closteroviridae*, the GLRaV-4 Ob genome possesses two large gene modules. One module is responsible for genome replication (MET, HEL and RdRp), whereas the other module includes five genes (p5, HSP70h, p60, CP, and p23) responsible for intercellular transport and virion assembly (Dolja et al. 2006). GLRaV-4 Ob's genomic organization and size are similar to viruses of subgroup II of the genus *Ampelovirus* (Martelli et al. 2012; Thompson et al. 2012). For example, the p23 ORF of GLRaV-4 Ob does not show any significant homology with CP ORFs and does not contain a closterovirus CP domain (pfam 01785). Thus, minor CP (CPm) is absent in GLRaV-4 Ob, as in all other GLRaV-4 strains (Naidu

et al. 2014). In contrast, members of subgroup I of the genus *Ampelovirus*, such as GLRaV-1 and GLRaV-3, all possess at least one CPm ORF in their genomes (Maree et al. 2013). Furthermore, GLRaV-4 Ob consistently grouped with viruses of the GLRaV-4 cluster in subgroup II of the genus *Ampelovirus* in phylogenetic analyses performed on the HSP70h gene (Fig. 6).

Despite sequence similarity with other GLRaV-4 strains, GLRaV-4 Ob has several genomic features that differentiate this strain from others. (i) This virus contains the smallest genome among viruses associated with grapevine leafroll disease. (ii) The length of the RdRp ORF in GLRaV-4 Ob is larger than that in other GLRaV-4 strains (Table 2). (iii) The p5 ORF of GLRaV-4 Ob overlaps the RdRp ORF, whereas other members of the GLRaV-4

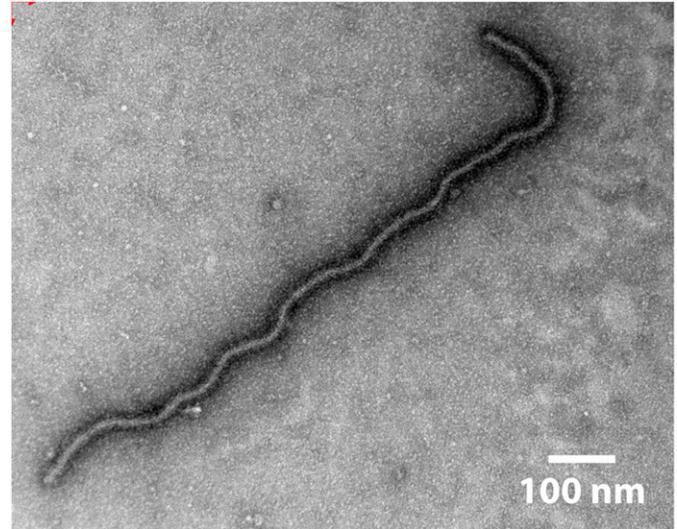


Fig. 4. Immuno-precipitation electron microscopy of *Grapevine leafroll-associated virus 4* strain Ob virions decorated with MAb37a.

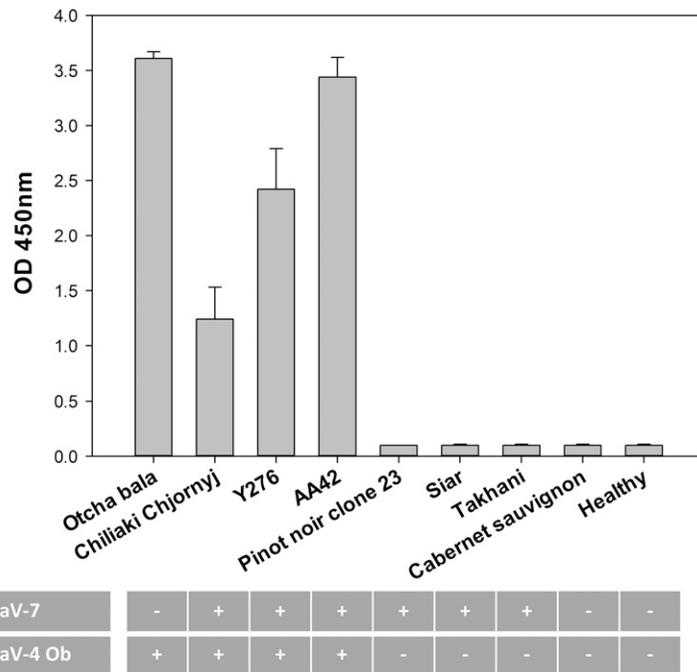


Fig. 3. Specific detection of *Grapevine leafroll-associated virus 4* strain Ob (GLRaV-4 Ob) in crude leaf extracts of different grapevine accessions by homologous double antibody sandwich enzyme-linked immunosorbent assays using MAb37a (four samples were analyzed for each accession, error bars represent standard deviations). reverse transcription-polymerase chain reaction results using GLRaV-4 Ob- and GLRaV-7-specific primer pairs are shown underneath for each accession (+, specific positive amplification; -, no amplification). Leaf extracts from GLRaV-4 strain 9-infected Cabernet sauvignon and a healthy grapevine were also tested. The absorbance was read after a 3-h incubation with the substrate.

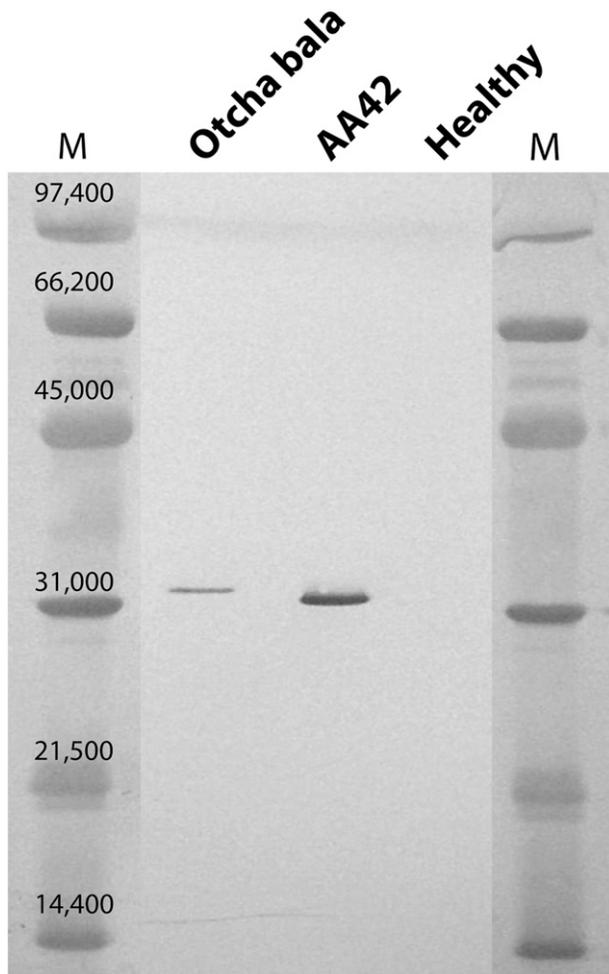


Fig. 5. Detection of the *Grapevine leafroll-associated virus 4* strain Ob by western blot analysis with MAb37a. M, molecular mass marker in Daltons.

cluster have an intergenic region between those two ORFs (Abou Ghanem-Sabanadzovic et al. 2012; Thompson et al. 2012;). Other ampeloviruses that share these features with GLRaV-4 Ob include *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) and *Pineapple mealybug wilt-associated viruses 1 and 3* (PMWaV-1 and PMWaV-3) (Melzer et al. 2008; Sether et al. 2009).

MAb37a was raised against accession Y276, which was initially thought to be infected only by GLRaV-7, and was therefore reported to be specific to GLRaV-7 (Rigotti et al. 2006). In this work, the double infection (GLRaV-7 and GLRaV-4 Ob) of the Y276 source was demonstrated. Western blot analysis indicated that MAb37a reacted to a structural protein with an apparent molecular weight of approximately 33 kDa (Fig. 5). CPs of other GLRaV-4 strains have been reported to have similar molecular weights ranging from 31 to 35 kDa as estimated by SDS-PAGE (Besse et al. 2009; Gugerli et al. 2009b; Rigotti et al. 2006). This molecular weight is larger than the expected molecular weight calculated from the CP amino acid sequences of GLRaV-4 strains (circa 30 kDa). However, such differences between theoretical molecular weight and SDS-PAGE estimates are common (Rubinson et al. 1997). MAb37a also reacted with the source AA42 (co-infected with GLRaV-7 and GLRaV-4 Ob), but not with the Pinot noir 23 source (infected only by GLRaV-7) as demonstrated by western blot (Fig. 5), ELISA (Fig. 3), and IPEM (data not shown). These two GLRaV-7 isolates have been sequenced, and their CPs share high amino acid sequence homology (identity: 96.3%, similarity: 99%) (Al Rwahnih et al. 2012; Jelkmann et al. 2012). Moreover, three additional accessions infected by GLRaV-7, but not by GLRaV-4 Ob, were also tested and they did not react with MAb37a. Therefore, common epitopes between GLRaV-4 Ob and GLRaV-7 do not seem to exist and MAb37a should be considered to be specific to GLRaV-4 Ob and not to GLRaV-7, as stated previously.

The GLRaV-4 Ob sequences determined in this study showed 93 to 98% identity with the previously reported GLRaV-4 Ru sequences at the amino acid level (Ito et al. 2013). Furthermore, the serological relatedness between GLRaV-4 Ob and GLRaV-4 Ru was demonstrated in this study using MAb37a in DAS-ELISA (Fig. 3). GLRaV-4 Ob and the published partial sequences of GLRaV-4 Ru1 and 2 are 87 and 88% identical at the nucleotide

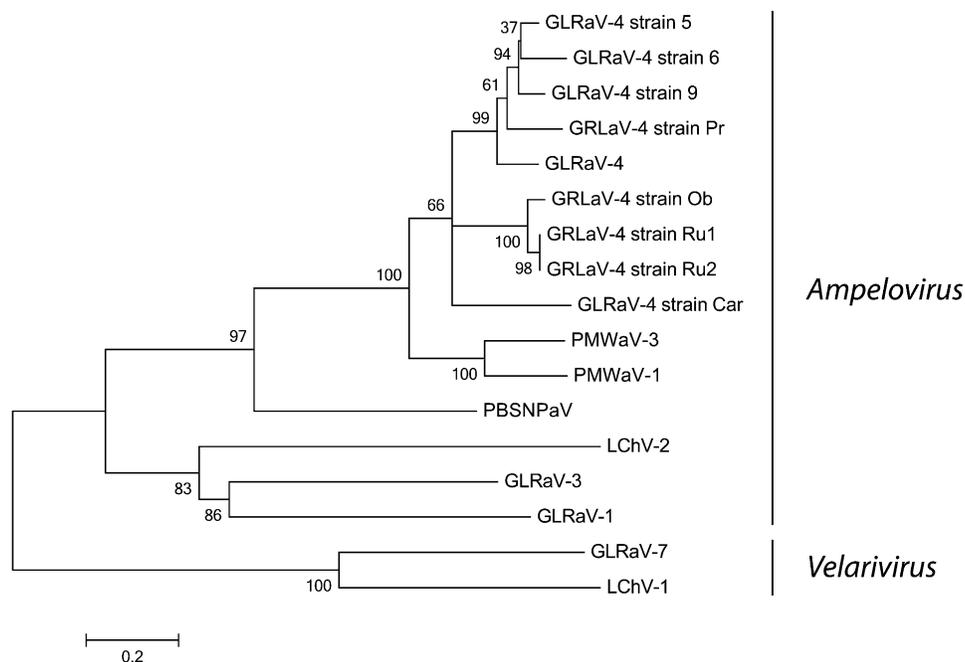


Fig. 6. Unrooted phylogram constructed using a multiple alignment of heat shock 70 protein homolog amino acid sequences from some members of the genera *Ampelovirus* and *Velarivirus*. The scale represents 0.2 amino acid substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

level, respectively. These two variants share a common epitope recognized by MAb37a; however, this epitope is not present in other GLRaV-4 strains because no cross-reactivity was observed in DAS-ELISA against GLRaV-4, GLRaV-4 strain 5, GLRaV-4 strain 6, and GLRaV-4 strain 9 (data not shown).

The serological data were in agreement with the molecular data and strongly supported the conclusion of Ito et al. (2013) that GLRaV-4 Ob, is similar to variant GLRaV-4 Ru, belongs to a distinct strain of the GLRaV-4 species. Furthermore, the amino acid identities between taxonomically relevant genes of GLRaV-4 Ob and other members of the GLRaV-4 species were between 68 to 78% (Table 2). The International Committee on Taxonomy of Viruses (ICTV) adopted an amino acid divergence threshold of 25% for RdRp, HSP70h and CP for the genus *Ampelovirus* (Thompson et al. 2012), making GLRaV-4 Ob a highly divergent strain. Therefore, GLRaV-4 Ob should be considered a more diverse strain of the GLRaV-4 species. GLRaV-4 strain Car is another example of a more diverged member of GLRaV-4 cluster (Abou Ghanem-Sabanadzovic et al. 2010).

A number of studies have utilized different starting materials for deep sequencing, including purified viral particles (Melcher et al. 2008), total RNA (Al Rwahnih et al. 2009; Wylie and Jones 2011), small interfering RNAs (Kreuze et al. 2009; Seguin et al. 2014), and double-stranded RNAs (Al Rwahnih et al. 2011, 2012; Coetzee et al. 2010). In this study, virus particles were first purified using ultracentrifugation before applying the deep sequencing techniques. This approach allowed us to obtain the complete genomic sequence of a closterovirus. The characterization of a new virus or strain is particularly tedious and laborious for woody crops due to low concentrations of the virus or due to the presence of inhibitors such as polyphenols that may interfere with virus purification and/or nucleic acid amplification techniques (Candresse et al. 2013). Furthermore, mixed infections and the extreme diversity of viruses infecting grapevines represent challenges for studying grapevine viruses. As previously reported (Al Rwahnih et al. 2009; Giampetruzzi et al. 2012; Studholme et al. 2011; Wu et al. 2010), the results presented here demonstrate the utility and value of applying deep sequencing technology to characterize new viral pathogens and to study viral disease etiology.

Serological and molecular data revealed that three grapevine accessions in our collection (Y276, Chiliaki Chjornyj, and AA42) are co-infected with GLRaV-4 Ob and GLRaV-7. Grapevines are prone to infection with several viruses and viral variants; thus, simultaneous infection by two or more viruses in the same grapevine plant is common (Al Rwahnih et al. 2009; Goszczynski 2013, Hu et al. 1990; Prosser et al. 2007; Sharma et al. 2011). For example, Chasselas 8/22 is co-infected with GLRaV-2, GLRaV-4

strain 5, GLRaV-4 strain 6, and an unidentified virus with isometric morphology (Gugerli et al. 1997; Abou Ghanem-Sabanadzovic et al. 2012). Previously, Chiliaki Chjornyj was reported to be co-infected with GLRaV-7 and GLRaV-4 Ru (Ito et al. 2013). Importantly, for the first time, our molecular and serological examinations of grapevine accession AA42 revealed a mixed infection of two members of the family *Closteroviridae*, GLRaV-7 and GLRaV-4 Ob.

GLRaV-7 was originally reported in a symptomless white-berried accession from Albania (AA42) that induced leafroll symptoms when grafted onto Cabernet Sauvignon indicators (Choueiri et al. 1996). Because no other closteroviruses were identified in AA42, GLRaV-7 was considered the causal agent responsible for leafroll symptoms (Martelli et al. 2012). However, different authors have reported that GLRaV-7 infections cause no or uncertain leafroll symptoms (Al Rwahnih et al. 2012; Avgelis and Boscia 2001; Morales and Monis 2007; Rowhani et al. 2012). Our findings suggest that the leafroll symptoms from the AA42 isolate may not be related to GLRaV-7 infection as reported previously but is due to GLRaV-4 Ob. To the best of our knowledge, no case of GLRaV-7 infection associated with leafroll symptoms has been reported where co-infection with other closteroviruses can be completely excluded. Pinot noir 23 is the only grapevine accession in which GLRaV-7 Swi is present as a unique closterovirus (Al Rwahnih et al. 2012), and this isolate does not induce any leafroll symptoms in Pinot noir and Cabernet sauvignon (Al Rwahnih et al. 2012). Because GLRaV-7 cannot be conclusively associated with symptomatic infection, this virus may not be a leafroll-causing agent. Our findings support the proposition made by Al Rwahnih et al. (2012) to replace the name GLRaV-7.

Interestingly, a situation similar to leafroll disease and GLRaV-7 may exist in cherries, another long-lived vegetatively propagated plant species. Little cherry viruses 1 and 2 are two species of the family *Closteroviridae* reported to be associated with little cherry disease (Jelkmann and Eastwell 2011). LChV-2 from the genus *Ampelovirus* induces typical little cherry disease symptoms in sweet and sour cherries (Jelkmann et al. 2008). In contrast, LChV-1, similar to GLRaV-7 belongs to the newly proposed genus *Velarivirus*; symptoms of LChV-1 infection are milder or absent because some isolates may not produce typical symptoms of little cherry disease (Katsiani et al. 2014; Matic et al. 2009; Schröder and Petruschke 2010).

In conclusion, this study describes a new virus that induces leafroll symptoms on 'Gamay' indicators. The serological and sequencing data reported here indicate that this virus belongs to subgroup II of the genus *Ampelovirus*. Therefore, we suggest the name *Grapevine leafroll-associated virus 4* strain Ob (GLRaV-4

TABLE 2. Amino acid (aa) sequence identities and the sizes of different genome products from viruses of the genus *Ampelovirus*^a

Virus	RNA-dependent RNA polymerase		Heat shock 70 protein homolog		Coat protein	
	Identity (%)	Size (aa)	Identity (%)	Size (aa)	Identity (%)	Size (aa)
GLRaV-4 Ob		526		533		261
GLRaV-4 AA42	98	*189	98	*173	93	261
GLRaV-4 strain Ru1	n.a.	n.a.	94	*520	93	261
GLRaV-4 strain Ru2	n.a.	n.a.	94	*520	98	261
GLRaV-4	74	517	71	533	77	272
GLRaV-4 strain 5	76	517	69	533	78	269
GLRaV-4 strain 6	78	517	69	533	76	269
GLRaV-4 strain 9	76	517	70	533	75	272
GLRaV-4 strain Pr	77	517	69	533	78	273
GLRaV-4 strain Car	76	516	68	534	77	267
PMWaV-1	58	525	59	509	57	257
PMWaV-3	56	525	60	533	67	262
PBNPaV	38	525	50	529	30	325
GLRaV-3	33	533	33	549	15	313

^a *Grapevine leafroll-associated virus 4* and 3 (GLRaV-4 and GLRaV-3), *Pineapple mealybug wilt-associated viruses 1* and 3 (PMWaV-1 and PMWaV-3), and *Plum bark necrosis stem pitting-associated virus* (PBNPaV). n.a. indicates not available. * indicates partial sequence.

Ob) for this virus. This work clearly demonstrates that two closteroviruses are co-infecting the AA42 grapevine, the accession from which GLRaV-7 was originally reported. The results presented here, together with previous reports of symptomless infection, suggest that GLRaV-7 is not associated with leafroll disease of grapevines. Future studies will be necessary to evaluate the phenotype of GLRaV-7 infections in grapevines definitively.

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