PARTIAL CHARACTERIZATION OF A NEW AMPELOVIRUS ASSOCIATED WITH GRAPEVINE LEAFROLL DISEASE

R. Alkowni, A. Rowhani, S. Daubert and D. Golino

Department of Plant Pathology, University of California, One Shields Avenue, Davis, CA 95616, USA

SUMMARY

A virus associated with leafroll symptoms in *Vitis vinifera* was selected for further analysis because it was undetectable in all reverse-transcriptase polymerase chain reaction (RT-PCR) tests available for Grapevine leafroll associated viruses (GLRaVs). Extracts of infected plant material were also negative in all enzyme-linked immunosorbent assays available for GLRaVs. Nonetheless, the unknown virus caused typical grapevine leafroll symptoms in the indicator host, *V. vinifera* cv Cabernet Franc. High molecular weight double-stranded RNA (dsRNA) purified from an infected vine appeared typical of the dsRNA recovered from plants infected by viruses in family *Closteroviridae*. RT-PCR was performed using this purified dsRNA with degenerate oligonucleotide primers designed to amplify a fragment from the heat shock protein 70 homologue (HSP70) from any closterovirus. Sequence information from the resulting RT-PCR products allowed the determination of a full length HSP70 sequence from the unknown virus for comparison with those of other GLRaVs. A specific RT-PCR assay based on primers developed from this sequence was negative for all GLRaVs tested, other than the unknown virus. The name Grapevine leafroll associated virus 9 (GLRaV-9) is tentatively proposed for the novel pathogen.

Key words: Grapevine leafroll associated virus-9, *Ampelovirus* genus, grapevine, grapevine virus detection, HSP70.

INTRODUCTION

To date, eight different viruses, all members of the family *Closteroviridae*, are known to be associated with leafroll disease in grapevine (*Vitis* spp). The disease alters fruit coloration, delays fruit maturation, and decreases yield by as much as 20-40% (Goheen and Cook, 1959; Woodrum *et al*., 1984; Goheen, 1988; Bovey and Martelli, 1992). Other symptoms include interveinal reddening (red grape varieties) or yellowing (white grape varieties) of late summer and fall leaves, downward rolling of leaves, and phloem disruption (Hoefort and Gifford, 1967; Weber *et al*., 1993). The symptoms of grapevine leafroll disease can resemble those caused by mechanical damage to the trunk and canes, by other diseases of the phloem tissue, and by arthropod damage, which complicates visual diagnosis (Weber *et al*., 1993).

The family *Closteroviridae,* includes viruses with flexuous rod-shaped virions of 1,250-2,200 nm in length that contain a positive sense, single stranded RNA of 15.5-19.3 kb (Martelli *et al*., 2002). The family comprises three genera: *Closterovirus* [type species: *Beet yellows virus* (BYV)], *Ampelovirus* [type species: *Grapevine leafroll associated virus 3* (GLRaV-3)] and *Crinivirus* [type species: *Lettuce infectious yellows virus* (LIYV)]. This is a new classification (Martelli *et al*., 2002) based on molecular and biological characteristics of family members.

Viruses in the genus *Ampelovirus* have a monopartite genome 16.9-19.5 kb in size that encodes a coat protein of 35-37 kDa, generally located upstream of the "coat protein minor" gene (Martelli *et al*., 2002)**,** and are transmitted by mealybugs. Viruses associated with leafroll disease of grapes (Karasev, 2000) have been collectively referred to as GLRaVs. GLRaV-1, -3, -4 and -5 are classified in the genus *Ampelovirus,* GLRaV-2 in the genus *Closterovirus*, and GLRaV-7 is unassigned (Martelli *et al.*, 2002). These viruses are serologically distinct. None is mechanically transmissible to herbaceous hosts, except for GLRaV-2, which is sap-transmissible to *Nicotiana benthamiana* (Boscia *et al*., 1995; Goszczynski *et al*., 1996). GLRaV-1 is transmitted by the scale insect *Parthenolecanium corni*, *Neopulvinaria innumerabilis* and by mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* (Sforza *et al*., 2000). GLRaV-3 is vectored by *Pseudococcus longispinus, Pseudococcus Viburny, Pseudococcus comstocki, Planococcus ficus*, *Planococcus citri, Planococcus calceolariae*, *Planococcus maritimus*, and *Pulvinaria vitis* (Belli *et al*., 1994; Martelli *et al.*, 1997; Golino *et al*., 2002; Nakano *et al*., 2003).

Among different GLRaVs only the genomic sequences

Corresponding author: A. Rowhani
Fax: +15.307.522132
E-mail: akrowhani@ucdavis.edu

of GLRaV-1, -2 and -3 are known close to full length (Habili *et al*., 1997; Abou-Ghanem *et al*., 1998; Ling *et al*., 1998; Zhu *et al*., 1998; Fazeli and Rezaian, 2000). The genomes of the rest of the GLRaVs have been partially characterized (Saldarelli *et al*., 1994; Routh *et al*., 1998; Turturo *et al*., 2000; Good and Monis, 2001) with the exception of GLRaV-6. GLRaVs -1, -3, and -5 are considered to be distinct species in the *Ampelovirus* genus (Martelli *et al*., 2002); GLRaV-4 is a tentative species.

Grapevine closteroviruses share genomic conserved regions [such as heat shock protein 70 phosphate motifs 1 and 2, HSP70-P1 and HSP70-P2 (Tian *et al*., 1996)], which allowed the design of the degenerate primers that are the current tools of choice for the detection of these viruses (Routh *et al*., 1998). Here we report the use of such degenerate primers in the cloning of HSP70 fragments from double-stranded RNA (dsRNA) prepared from grapevines infected with isolate GLRV118 (Golino, 1992). We identify this isolate as a putative new ampelovirus, tentatively named GLRaV-9, and describe the sequence of its HSP70 open reading frame (ORF) and adjoining sequences. We have used that sequence information to develop an RT-PCR assay for the specific detection of this novel virus.

MATERIALS AND METHODS

Virus source, detection, and dsRNA analysis. GLRaV-9 was isolate GLRV118 (Golino, 1992), maintained in *V. vinifera* L. hybrid Helena (Zinfandel x Refosco) in the field and in the pots in the greenhouse. It was chip bud inoculated into *V. vinifera* cv Cabernet Franc. The inoculated host plants were planted and maintained in the field for 1.5 years before the symptoms were observed and recorded. The source vine was also tested by ELISA for GLRaV-1 to -6 and by RT-PCR for GLRaV-1 to -7 (RT-PCR and serological detection methods are not available for GLRaV-6 or -8, respectively). For the detection of GLRaV-1, -2 and -6 by ELISA, a kit obtained from Bioreba (Reinach, Switzerland) was used following the manufacturer's protocol. For GLRaV-3 and -4 assays, polyclonal antibodies produced at University of California (Davis, USA) were used in a F(ab')2 ELISA system (Rowhani, 1992), and for GLRaV-5 detection, antibodies were from Agdia (Elkhart, IN, USA) following the manufacturer's protocol. The samples for ELISA tests were prepared by collecting randomly at least six leaf petioles from each vine and extracting them as described previously (Rowhani, 1992). These samples were collected either in the fall from the vines grown in the field or in any season from greenhouse-grown plants. For RT-PCR sample preparation and PCR amplification, a previously described method was used (Rowhani *et al*., 2000a). All tests were repeated at least two times.

dsRNA was extracted from cortical scrapings from mature canes and from leaf petioles as described by Valverde *et al.* (1990). The dsRNA preparation was analyzed in 6% polyacrylamide gel after DNase and RNase digestion as described previously (Saldarelli *et al*., 1996) and bands were visualized by silver staining (Sambrook *et al*., 1989).

cDNA cloning and sequencing. For cDNA synthesis and cloning, RT-PCR was used in the presence of degenerate primers designed to amplify a 550-650 base pair (bp) fragment from the HSP70 homologue of closteroviruses (Tian *et al*., 1996). These primers included HSP-P1, GGNTTAGANTTCGGNACNAC designed on phosphate motif 1 and HSP-P2, TCAAANGTNCCNCCNCCNAA on phosphate motif 2. Briefly, cDNAs were synthesized using dsRNA as templates as previously described (Jelkmann *et al*., 1989; Habili and Rezaian, 1995) with some modifications. Approximately 50 ng purified dsRNA was denatured in 20 mM methyl mercuric hydroxide at room temperature for 10 min in the presence of 350 ng random sequence primers. Four hundred units of Superscript II RT (Invitrogen Life Technologies, Carlsbad, CA, USA) were used for the first strand cDNA synthesis at 37°C for 1 h. The PCR was performed by adding 5 µl of the cDNA to 95 µl of PCR mix (1xPCR buffer II, 200 ng of the degenerate primers HSP-P1 and HSP-P2, 3 mM $MgCl_2$, 0.2 mM dNTP, and one unit of Ampli*Taq* DNA polymerase). PCR cycles were as described by Tian *et al.* (1996).

PCR products were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) and analyzed in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA). The expected DNA fragments were recovered from the gel and purified by QIAGEN Gel Purification Kit and ligated to pCR4-TOPO (Invitrogen Life Technologies, Carlsbad, CA, USA) using the TA cloning Kit following the manufacturer's recommendation. The ligated plasmids were then transformed into One Shot TOPO10 *E. coli* competent cells. Selected colonies were screened and their plasmids were extracted by QIAGEN miniprep extraction kit (Qiagen Inc., Valencia, CA, USA). The plasmids were digested with *Eco*RI restriction enzyme (Promega, Madison, WI, USA) and several clones containing the dsDNA insert (ca 600 bp in length) were selected and sequenced in both directions using the automated sequencing facility (DBS) at the University of California (Davis, USA). The sequence data were processed using the Chromas program (Techneylysium Pty. Ltd., Tewantin, Qld, Australia) and compared. Database searching for similarity to any amino acid sequences found in that database was conducted by BLAST search programs of the National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1997).

The sequence obtained by PCR amplification was used for designing specific primers for downstream and

upstream sequence extension, upon the purified dsRNA templates. Extracted dsRNAs were denatured by methyl mercuric hydroxide as previously described in the presence of the specific primers. To obtain a cDNA library, a cDNA synthesis kit was used following the protocol provided by the manufacturer's (Invitrogen Life Technologies, Carlsbad, CA, USA) with some modification described previously (Zhang and Rowhani, 2000). The produced dsDNA was ligated to pCR4-TOPO (Invitrogen Life Technologies, Carlsbad, CA, USA) by using the TA cloning Kit following the manufacturer recommendations for electroporation transformation. The ligated plasmids were transformed into competent cells (ElectroMax DH10B) by using a micropulser electroporater (Bio-Rad Laboratories Hercules, CA, USA). This reaction was repeated for further sequence extension of the viral genomes in both directions. Clone selection and sequencing were followed as described above.

Sequence analysis. The full length HSP70 ORF of GLRaV-9 was sequenced and analyzed using the available package programs from the University of Wisconsin Genetics Computer Group (GCG) (Devereux *et al*., 1984). The deduced sequences were compared to closterovirus and ampelovirus sequences available in databank of NCBI. These viruses included: GLRaV-1 (AF195822); GLRaV-2 (AF039204); GLRaV-3 (AF037268); GLRaV-4 (AF039553); GLRaV-5 (AF233934); GLRaV-7 (Y15987); BYV (NC001598); LIYV (NC003618); *Citrus tristeza virus* (CTV) (U16304); *Potato yellow vein virus* (PYVV) (AF150984); *Little cherry virus 1* (LChV-1) (Y10237); and *Pineapple mealybug associated virus 1* (PMWV-1) (AF414119). HSP70 of *Daucus carota* (X60088) was used as an outgroup.

Phylogenetic analysis were done by PileUp aligning program and with the assistance of the PAUP*4.0 programs (Sinauer Associates, Inc., Sunderland, MA, USA). The ORFs of GLRaV-9 were identified from the available sequences and the putative protein translation and molecular analysis were obtained by using the DNASIS Max program package (Hitachi software Engineering Co., Ltd., Wembley, Middlesex, UK). The program GAP was used to obtain the best-fit alignments of the nucleotide and amino acid sequences of RdRp and HSP70 of GLRaV-9 with those of some members of the family *Closteroviridae*.

Western blot. Samples were prepared from grapevines infected with GLRaV-4, -5 or -9 for Western blot analysis as described previously (Parker *et al.*, 1989) with some modifications. Proteins were transferred to nitrocellulose membrane using trans-Blot cell (Bio-Rad Laboratories, Hercules, CA, USA) at 150 mA constant for 15 h (overnight). After transfer, the membranes were blocked by washing for 30 min in 0.02 M potassium phosphate buffer, pH 7.3, containing 0.115 M NaCl and 0.1% Tween 20. The membranes were then incubated at room temperature for 1 h with specific polyclonal antibodies to GLRaV-4 (produced at University of California, Davis, USA) and GLRaV-5 (Agdia, Elkhart, IN, USA). Membranes were washed with TBST buffer (2 mM Tris-base, 50 mM sodium chloride, 0.05% Tween 20, pH 7.4) and incubated with biotin-labeled goat anti-rabbit antibody and stained using the Immuno-Blot kit following the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

Primer selection and virus survey. The sequence data for GLRaV-9, was used to design the specific primers LR9-F, 5'-CGGCATAAGAAAAGATGGCAC-3' and LR9-R, 5'-TCATTCACCACTGCTTGAAC-3' able to amplify a segment of 393 bp. The primers were used in RT-PCR tests for virus detection in different grapevine varieties. Sample preparation and one tube RT-PCR were as described previously (Rowhani *et al*., 2000a) with annealing temperature of 58°C for 30 sec in each cycle. PCR products were analyzed in 1.5% agarose gel electrophoresis in TAE buffer and visualized by ethidium bromide staining. The specificity of the amplified products was verified by sequencing or by dot blot hybridization using non-radioactive DIG-riboprobe, specific to a portion of virus HSP70 sequence, that was prepared from the cloned PCR products. The opposite polarity riboprobes were generated by using SP6 RNA polymerase and the DIG-RNA labeling kit (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. For virus survey, leaf petiole samples were collected in the fall from different vineyards in California and tested by RT-PCR as described above using GLRaV-9 specific primers. Total of 230 vines were tested in this survey.

RESULTS

Symptomatology and virus detection. GLRaV-9 induced symptoms on *V. vinifera* cv Cabernet Franc characteristic of grapevine leafroll disease, including faint interveinal reddening of the leaves. Leaf petiole samples collected from the source plant tested negative in ELISA for GLRaV-1 through -6. The sample was also negative by RT-PCR for GLRaVs-1 to -7 (excluding GLRaV-6, due to the absence of specific detection primers), and for a Red Globe strain of GLRaV-2 (GLRaV-2-RG, unpublished) formerly named as Grapevine rootstock stem-lesion associated virus (Rowhani *et al*., 2000b). DsRNA analysis of GLRaV-9-infected tissues revealed a high molecular weight band similar to and typical of closterovirus and ampelovirus dsRNA (Fig. 1A). The use of degenerate primers HSP-P1 and HSP-P2 in RT-PCR, using the dsRNA from this isolate as template, amplified a fragment of 590 bp (Fig. 1B) from the HSP70 region.

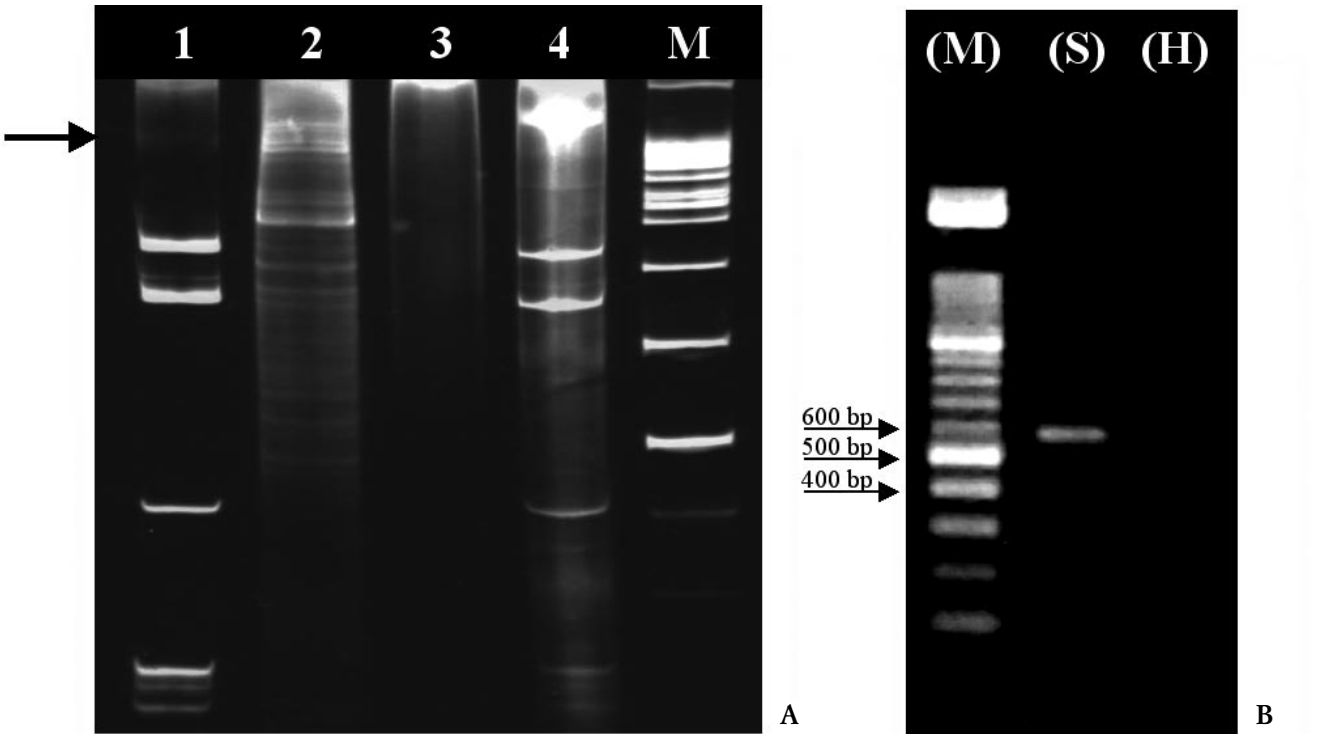

Fig. 1. A) 6% polyacrylamide gel electrophoresis analysis of double-stranded RNA (dsRNA) extracted from *Vitis vinifera*. Lane 1: *Cucumber mosaic virus* dsRNA marker; lane 2: isolate GLRV118; lane 3: healthy grapevine; lane 4: C*itrus tristeza virus* dsRNA marker; Lane M: 1kb DNA ladder. **B)** 1.5% agarose gel electrophoresis analysis of purified reverse-transcription polymerase chain reaction products amplified from isolate GLRV118 using degenerate primers HSP-P1 and P2. A *ca.* 600 base pair dsDNA fragment was detected (Lane S). Lanes H and M are healthy grapevine, and DNA size marker XIV (Roche, Manheim, Germany), respectively.

Cloning, sequencing, and sequence analysis. Seven clones from the RT-PCR products of HSP-P1/P2 products were selected and sequenced in both directions. All the cloned fragments had identical 590 bp sequences that encoded a sequence of 196 amino acids.

Primers specific for GLRaV-9 were designed, based on the HSP70 sequence segment determined from the above clones. The resultant subclones covered more than 3 kb of the virus genome (Fig. 2), including the entire coding region for the RNA-dependent RNA polymerase (RdRp) reading frame (ORF 1b), ORFs 2 and 3, and all of HSP70 ORF4. The nucleotide sequence has been assigned GenBank accession number (AY072797).

ORF1b, a sequence homologous to the RdRp gene of other closteroviruses, was 1,194 bp long (including the initiation codon), with a molecular mass of 44.5 kDa, calculated for the implied translation product (using the DNASIS Max package program). The nucleotide sequence of this frame overlapped with the terminus of the preceding ORF, including its stop codon (ATGTT*TAG*CGTA).

The nucleotide sequences of the RdRp and HSP70 genes of GLRaV-9 were compared with those of a number of other viruses in the family *Closteroviridae* (Table 1). ORF2 starts on the 15th nucleotide after ORF1 ends. It is 255 bp in length and encodes 85 amino acids with a predicted *Mr* of about 10 kDa. ORF3 (a small ORF unique to ampeloviruses) was 138 nucleotides long, encoding 46 amino acids with a predicted *Mr* of about 5 kDa.

The HSP70 sequence of GLRaV-9 started on the 7th nucleotide after the stop codon of the previous frame, and contained 1,599 nucleotides. Its stop codon overlapped with the beginning of the next ORF, which has its start codon within the end of the HSP70 frame. The HSP70 protein has a predicted *Mr* of 58 kDa. Similarity searching against the NCBI-BLASTN nucleotide database [GCG; (Altschul *et al.*, 1997)], demonstrated a significant homology ($P < e^{-10}$) between the HSP70 nucleotide sequence of GLRaV-9 and that of GLRaV-5. Sequence comparison for best-fit alignment (Henikoff and Henikoff, 1992) of GLRaV-9 with GLRaV-5 revealed homology of 78% and 88% within the HSP70 gene for their nucleotide and amino acid sequences, respectively (Table 1). Amino acid alignments using the PileUp program (GCG) comparing the GLRaV-9 HSP70 translation sequence with those of other viruses associated with grapevine leafroll disease (GLRaV-1 to 5, and GLRaV-7), revealed numerous regions of peptide homology (Fig. 3). Amino acid alignment of the con-

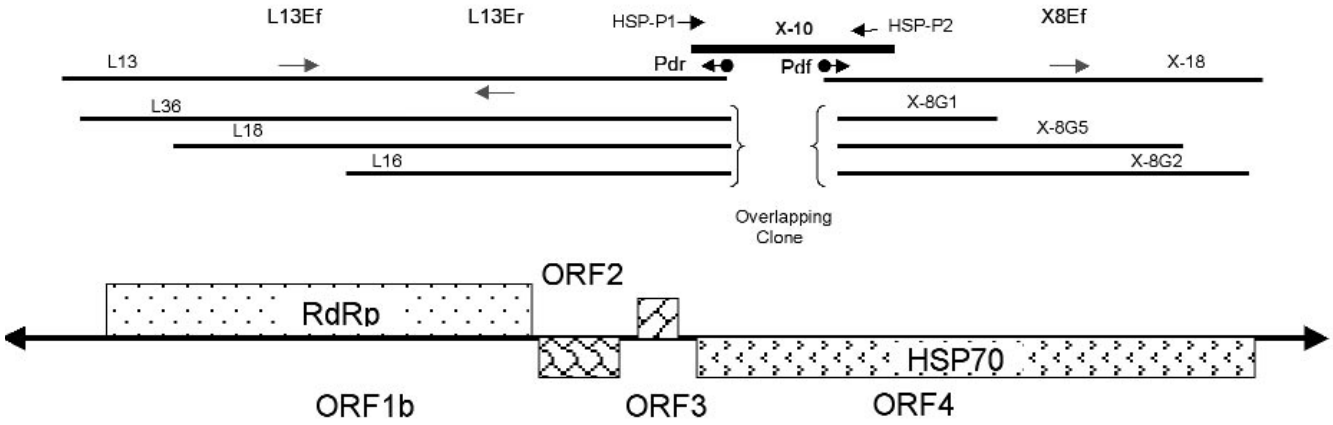

Fig. 2. Schematic of the genomic organization of the cloned section of Grapevine leafroll associated virus-9, and the cloning strategy used to cover the open reading frames (1b, 2, 3 and 4). Two major clones (L13 and 8), in addition to the RT-PCR amplified portion in between motif 1 and motif 2 on the genome HSP70 covered the span. Also, other overlapping clones are listed. Small arrows indicate the primer binding sites.

Table 1. Sequence comparison for best-fit alignment of the deduced nucleotide sequence of *Grapevine leafroll associated virus*-9 (GLRaV-9) RNA-dependent RNA polymerase (RdRp) and heat shock protein 70 (HSP70) genes with other members of the *Closteroviridae.* Nucleic acid and amino acid alignments were done by the GAP program, using different Genebank virus sequences. Sequences are: *Beet yellows virus* [BYV (NC_001598)], *Citrus tristeza virus* [CTV (U16304)], GLRaV-1 (AF195822), GLRaV-2 (AF039204), GLRaV-3 (AF037268), GLRaV-4 (AF039553), GLRaV-5 (AF233934), GLRaV-7 (Y15987), *Little cherry virus* [LChV (Y10237)], *Lettuce infectious yellows virus* [LIYV (U15440) and (NC003618)], *Pineapple mealy bug associated virus-1* [PMWaV-1 (AF414119)], and *Potato yellow vein virus* [PYVV (AF150984)].

Virus	RdRp		HSP70	
	Nucleic acid identity (%)	Amino acid identity (%)	Nucleic acid identity (%)	Amino acid identity (%)
GLRaV-5	n.a. [a]	n.a.	78.4	88.5
GLRaV-4	n.a.	n.a.	73.8	87.3
PMWaV-1	60.1	59.7	59.4	60.5
GLRaV-3	46.1	31.7	45.8	36.8
GLRaV-7	n.a.	n.a.	43.4	36.7
GLRaV-1	44.8	34.1	45.5	34.2
PYVV	n.a.	n.a.	47.0	32.7
GLRaV-2	45.2	32.8	44.1	32.6
BYV	44.2	30.4	44.5	31.3
LIYV	41.1	25.9	43.0	29.7
LChV	41.6	26.7	44.9	28.3
CTV	44.0	32.5	43.6	27.4

[a] n.a. = Sequence of this part of the genome is not available in GenBank.

served sequences located between the HSP70 phosphate motif 1 and motif 2 of GLRaV-9 with other GLRaVs, showed (94%) identity to the GLRaV-5 and (87%) to GLRaV-4 (Table 1). These similarities exist also between the two distinct viruses GLRaV-4 and GLRaV-5 as identity among their amino acid sequence reaches to 90% in the same region of their genomes.

Detection of GLRaV-9 by ELISA, RT-PCR and Western Blot. Extracts of plants infected with GLRaV-9 were negative in ELISA using antisera to GLRaV-1 to 6. These tests were repeated at least twice to confirm the result. Samples prepared from a plant infected with GLRaV-9 (isolate GLRV118) tested negative in RT-PCR when PCR primers specific for GLRaV-1, -2, -3, -4, -5 and -7 were used. Conversely, specific RT-PCR primers used for the detection of GLRaV-9 failed to detect any of the above mentioned grapevine leafroll associated viruses (Fig. 4a; experiments repeated at least twice). Furthermore, Western blot analysis using the GLRaV-5 and GLRaV-4 specific IgG only detected their specific SDS-denatured proteins (Fig. 4b; analysis repeated twice).

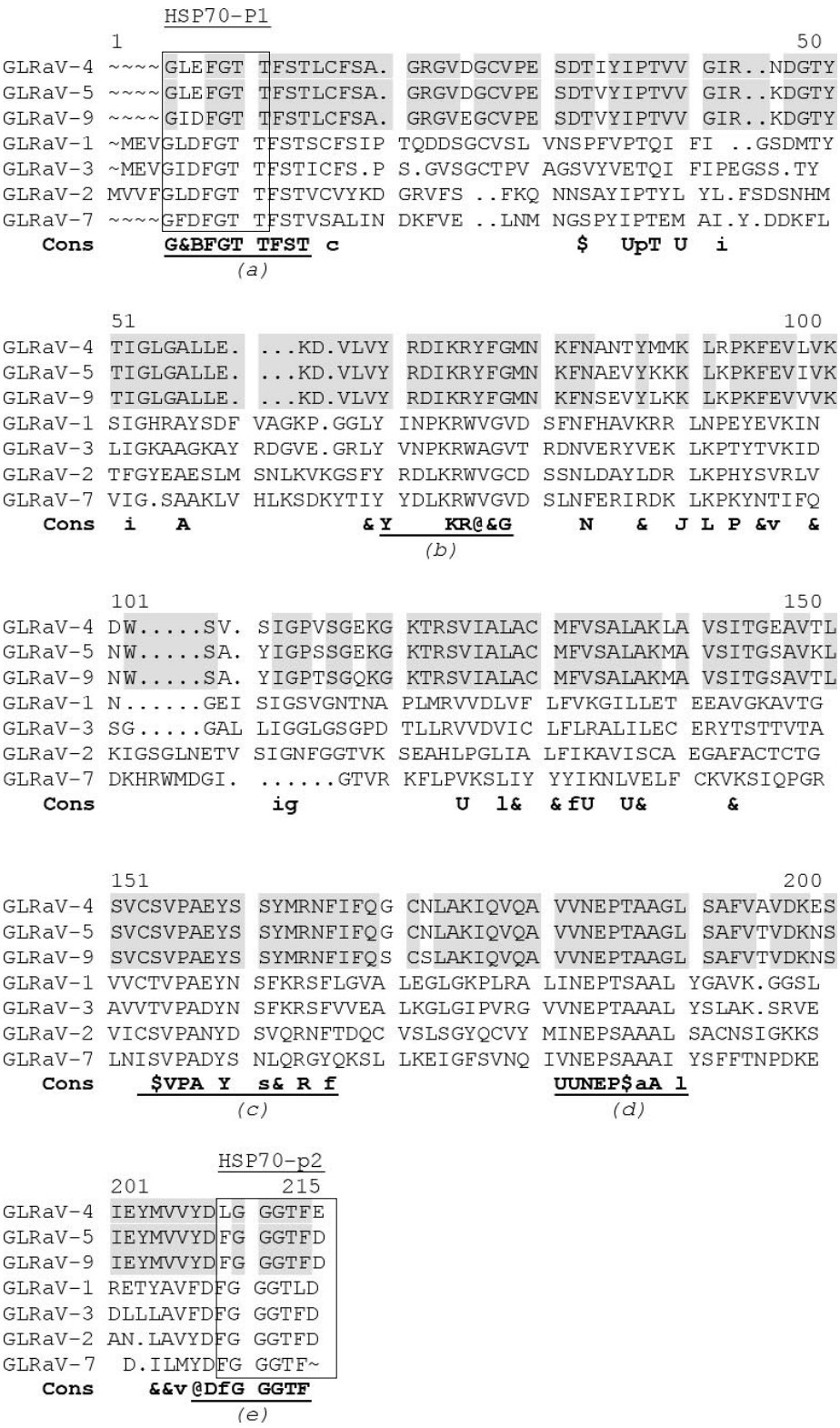

Fig. 3. Alignment of conserved amino acids in the sequence of Grapevine leafroll associated virus-9 (GLRaV-9) compared with GLRaV- 1, -2, -3, -4, -5, and -7 sequences, between the phosphate 1 and 2 motifs of their heat shock protein 70 genes (boxed). Motifs conserved in this region of the closteroviruses genome are indicated by above lined letters *a*, *b*, *c*, *d* and *e*. The alignment was done by the PileUp program (University of Wisconsin, Genetics Computer Group), showing the consensus amino acids (Cons) in bold and the conserved ones between deduced sequence and GLRaV-4 and GLRaV-5 as shaded. The consensus (lower case) represents amino acids, which are conserved in six sequences, while (UPPER CASE) are amino acids conserved in all sequences. The consensus (U) indicates bulky aliphatic residues [I, L, V, or M]; ($) for [S or T]; (J) is for positively charged residues [K or R], (B) indicates negatively charged [D or E], @ aromatic residues [F, Y, or W], (&) bulky hydrophobic residues [I, L, V, M, F, Y, or A]; gaps are indicated by dots.

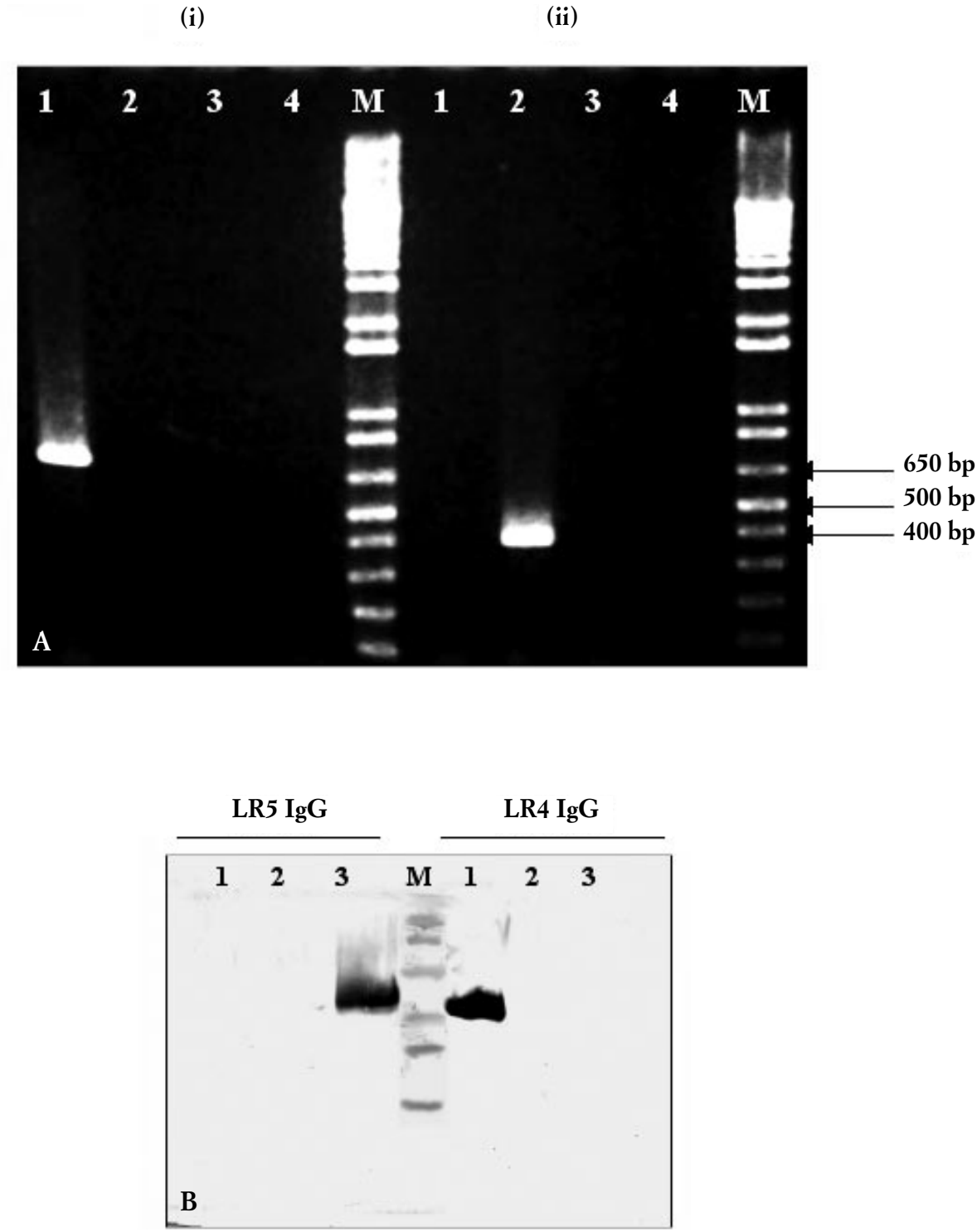

Fig. 4. A) Agarose gel electrophoresis analysis of reverse-transcription polymerase chain reaction tests for specific detection of *Grapevine leafroll associated virus*-5 (GLRaV-5) (Panel i) and GLRaV-9 (Panel ii): from GLRaV-5 infected grapevine (Lane 1), GLR118 isolate (Lane 2), healthy grapevine (Lane 3), and blank (Lane 4). Lane M is 1kb DNA ladder (GIBCO BRL). The (*ca* 393 bp) fragment of the HSP70 gene was only amplified from GLRaV-9 source GLRV118 (Panel ii, Lane 2), using the primer pair of LR9-F and LR9-R. Likewise, specific primers to GRLaV-5 only amplified the expected fragment from a GLRaV-5-infected vine (Panel i, Lane 1). **B)** Western blot analysis of GLRaV-9 (Lane 2), GLRaV-4 (Lane 1) and GLRaV-5 (Lane 3) proteins against GLRaV antisera of GLRaV-5 (Plantest-ELISA, France) and the IgG of GLRaV-4 in two separate analysis. M is BioRad prestained standards markers containing phosphorylase B (107 kDa), bovine serum albumin (90 kDa), ovalbumin (49.6 kDa), carbonic anhydrase (35 kDa), soya bean trypsin inhibitor (27.8 kDa) and lysozyme (20.2 kDa).

Phylogenetic comparison. A phylogenetic tree generated using the nucleotide sequences for the HSP70 showed that GLRaV-9 is closely related to GLRaV-4 and -5 and more distantly related to GLRaV-2 and -7 (Fig. 5). However, GLRaV-9 clustered with mealybug-transmitted viruses PMWaV-1, GLRaV-3 and GLRaV-1.

Virus survey. A limited survey indicated that GLRaV-9 was present in other *V. vinifera* cultivars in Californian vineyards. A total of 5 vines from 230 vines tested (2%) were positive for the virus. All five infected vines were *Vitis* species and none of them were positive for GLRaV-5. The results obtained by RT-PCR revealed the spread of the virus in different grape growing areas in Australia (Habili and Rowhani, 2002).

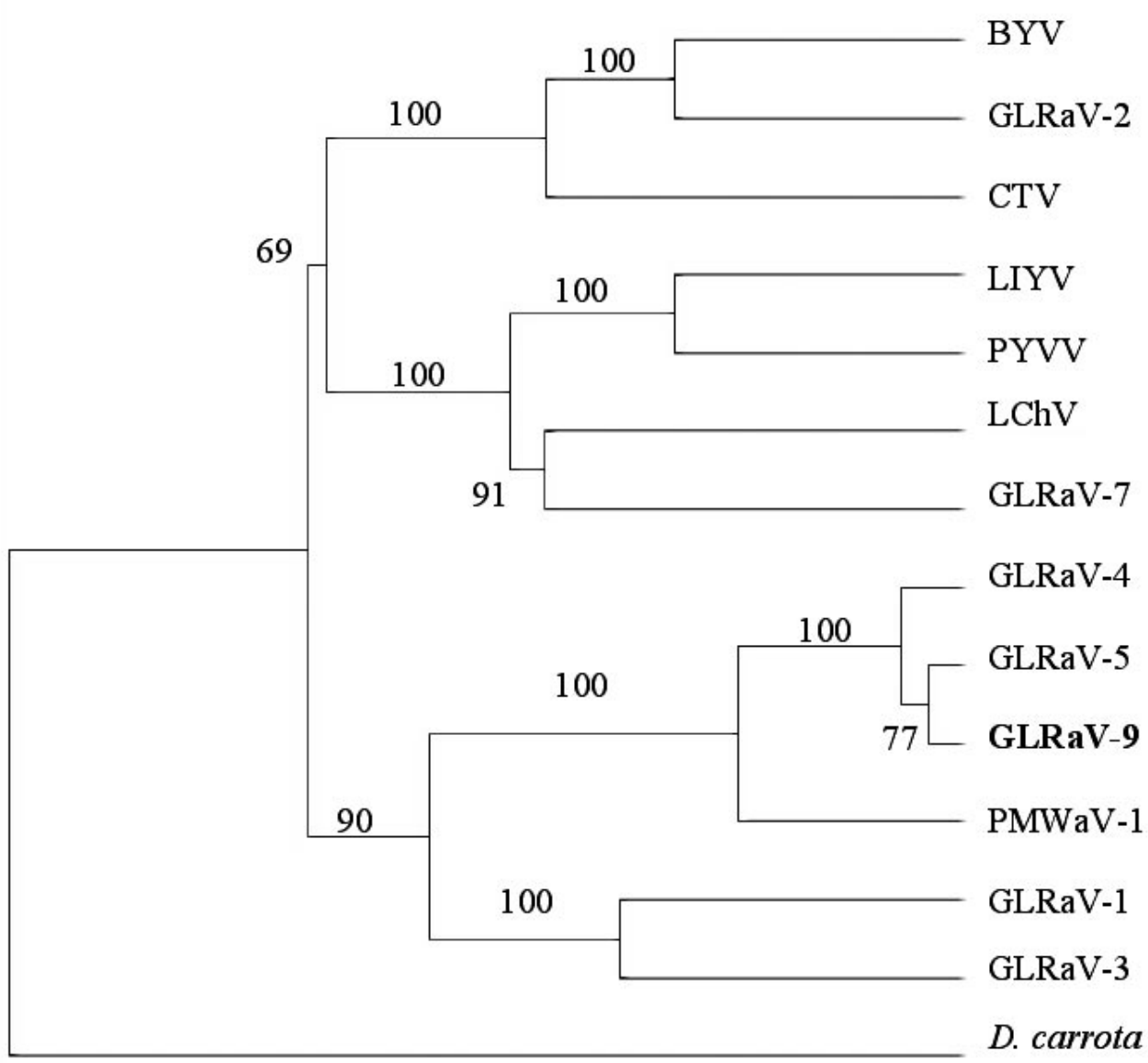

Fig. 5. Phylogenetic analysis showing the relationships of Grapevine leafroll associated virus -9 (GLRaV-9) with members of family *Closteroviridae* based on the heat shock protein 70 (HSP70) homologue nucleotide sequences conserved between the phosphate 1 and 2 motifs. The tree was generated by PileUp package programs available from the University of Wisconsin Genetics Computer Group with the assistance of the PAUP*4.0 programs. Horizontal distances are proportional to sequence distances and vertical distances are arbitrary. The dendrogram was Bootstrapped 100 times (present scores are on relevant horizontal branches). Database accession numbers used in the analysis are: GLRaV-1 (AF195822); GLRaV-2 (AF039204); GLRaV-3 (AF037268); GLRaV-4 (AF039553); GLRaV-5 (AF233934); GLRaV-7 (Y15987); *Beet yellows virus* (BYV) (NC001598); *Lettuce infectious yellows virus* (LIYV) (NC003618); *Citrus tristeza virus* (CTV) (U16304); *Potato yellow vein virus* (PYVV) (AF150984); *Little cherry virus 1* (LChV-1) (Y10237) and *Pineapple mealybug associated virus -1* (PMWaV-1) (AF414119). HSP70 of *Daucus carota* (X60088) was used as an outgroup.

DISCUSSION

Symptom expression in the grapevine leafroll indicator host *V. vinifera* cv Cabernet Franc, together with dsRNA profiles, and the presence of an HSP70 gene homologue [a gene conserved in all members of the family *Closteroviridae* (Tian *et al*., 1996)] suggested that GLRaV-9 was a previously undescribed grapevine leafroll-associated virus (GLRaV). The sequence of the GLRaV-9 HSP70 gene was used to develop a specific RT-PCR assay for the detection of this new virus. The new assay did not detect viruses in tissue samples infected with GLRaVs-1 to -7.

Phylogenetic analysis generated on the HSP70 sequence (Fig. 5), grouped GLRaV-9 together with GLRaV-5, GLRaV-4, PMWaV-1, GLRaV-1, and GLRaV-3, all of which are ampeloviruses. These phylogenetic data concur with published data (Sim *et al*., 2003) to suggest that the transmission of this virus is by mealybugs. Further studies will be required to fully describe the etiology of GLRaV-9.

Comparative analysis of the HSP70 nucleic acid sequences of the phosphate motif 1 and 2 regions shows that GLRaV-9 is less similar to GLRaV-4 (73.8% homology) than GLRaV-4 is to GLRaV-5 (77.5% homology). GLRaV-9 was found to have two ORFs between its putative RdRp and the HSP70 gene, similar to the genomic organization of GLRaV-3. The RdRp of GLRaV-9 is the smallest GLRaV RdRp reported to date.

Further evidence indicating that GLRaV-9 belongs to the genus *Ampelovirus* was derived from database searches for peptide similarity in NBCI-BLASTP [GCG, (Altschul *et al*., 1997)]. This study revealed a relatively high sequence homology (54% identity) between the deduced amino acid sequences of the GLRaV-9 RdRp and that of the ampelovirus PMWaV-1 (Table 1). This pair was more similar than the GLRaV-9 sequence was with sequences of any other GLRaV.

The new isolate was serologically distinct when tested by ELISA using antibodies produced against GLRaVs-1 to -6. In Western blot experiments using polyclonal antibodies to GLRaVs-4 and -5 (which, among the ampeloviruses, are most closely related to GLRaV-9 as determined by amino acid alignments between HSP70 genes; see Fig. 3) only the homologous control viruses reacted positively (Fig. 4b). In a different RT-PCR experiment using specific primers for the detection of GLRaVs-1 to -5, and -7, again only the original viruses were amplified (RT-PCR detection methods for GLRaVs-6 and -8 are not yet available).

We are in the process of characterizing the prevalence of GLRaV-9 in infected vineyards. Leafroll infections commonly include multiple GLRaVs (Martelli *et al*., 1997). Only the known viruses will be detected among them, using the diagnostic tools presently available. With the advent of the new RT-PCR assay described here, GLRaV-9 originally isolated in the U.S. has been discovered in Australia (Habili and Rowhani, 2002). With the continued broadening of our specific diagnostic tool kit for GLRaV species, we will be better able to determine the complexity of leafroll virus infections in vineyards, and the relative importance of each virus to those infections.

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