SHORT COMMUNICATION

PARTIAL CHARACTERISATION OF A GRAPEVINE LEAFROLL-ASSOCIATED VIRUS ISOLATED FROM AN INFECTED CYPRIOT VINE OF cv. MAVRO

T. Elbeaino[1], F. Numic[1], M. Digiaro[1], S. Sabanadzovic[2] and G.P. Martelli[3]

[1] Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy
[2] Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA
[3] Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Unità Organizzativa di Bari, Via Amendola 165/A, 70126 Bari, Italy

SUMMARY

A grapevine accession of cv. Mavro from Cyprus showing mild leafroll symptoms and containing closterovirus-like virus particles failed to react with commercial antisera to *Grapevine leafroll-associated virus 1*, *-2*, *-3*, *-5* and *-7*. The virus, denoted GLRaV-Cyp1, was not mechanically transmissible to herbaceous hosts but was successfully transferred by chip-bud grafting and through *Planococcus ficus* to healthy rooted cutting of cv. Cabernet sauvignon in which it induced leafroll symptoms as mild as those seen in the mother vine. Three different fragments of the viral genome, corresponding to the heat shock protein 70 homologue (HSP70h), major coat protein (CP) and p23 genes were amplified using degenerate and specific primers, cloned and sequenced. In a comparative analysis, GLRaV-Cyp1 showed amino acid sequence identity never higher than 86% with the HSP70h and 70% with CP and p23 genes of other GLRaVs species and strains, with the exception of a GLRaV isolate from a Greek grapevine, with which it showed 94% identity. In phylogenetic trees constructed with sequences of HSP70h, CP and p23 genes, GLRaV-Cyp1 consistently grouped in a cluster comprising sequences of the "type strains" of GLRaV-4, -5, -6, and -9 and of several other viral isolates regarded as molecular variants of one or more of the above species. This supports the notion that GLRaV-Cyp1 may not be a new ampelovirus species. In a small-scale survey carried out by PCR on a collection of vines from ten Mediterranean countries, GLRaV-Cyp1 was detected in 5 out of 121 vines tested.

Key words: Grapevine, ampeloviruses, leafroll, biological properties, molecular variants sequencing. epidemiology.

Corresponding author: T. Elbeaino
Fax: 39.080.4606327
E-mail: elbeaino@iamb.it

During routine field inspection of the grapevine collection of the Mediterranean Agronomic Institute of Bari (IAMB) for the presence of leafroll-associated viruses (GLRaVs), a vine of cv. Mavro from Cyprus showing mild leafroll symptoms was observed. This vine was examined for the presence of grapevine leafroll-associated virus-specific double-stranded RNAs (dsRNAs) and multiple extraction experiments were conducted according to Dodds (1993). Briefly, *ca.* 30 g cortical scrapings were ground in a mortar with liquid nitrogen and extracted with two rounds of phenol-chloroform treatment. After CF11 cellulose chromatography, single-stranded nucleic acids were digested as described by Saldarelli *et al.* (1994). After phenol/chloroform extraction, and centrifugation at 9,000 rpm for 10 min, dsRNA was ethanol-precipitated from the supernatant and electrophoresed in 5% TAE polyacrylamide gel (Sambrook *et al.*, 1989). Electropherograms showed a dsRNA band *ca.* 17 kbp in size (not shown), a value in the range of those expected for members of the genus *Ampelovirus* (Martelli *et al.*, 2005).

To prove that the Cypriot vine was not infected by one or more of the known Grapevine leafroll-associated viruses (GLRaVs), the presence of GLRaV-1, -2, -3, -5, -6 and -7 was checked with commercial ELISA kits from Agritest (Italy) and Sediag (USA). Cortical scrapings from mature canes of Cyp-1, healthy and positive controls (infected vines for each of the above mentioned viruses) were used as antigen sources. No reaction was observed with any of the antisera. RT-PCR was employed for detecting other grapevine leafroll associated viruses not tested by ELISA, i.e. GLRaV-4 (Routh *et al.,* 1998) and GLRaV-9 (Alkowni *et al.*, 2004). Also for these two viruses the result was negative. However, when a set of degenerate primers designed on conserved regions of the heat shock protein 70 homologue (HSP70h) gene of viruses of the family *Closteroviridae* (Tian *et al.,* 1996) was used for PCR, a product of 620 bp in size was amplified (Fig. 1), suggesting that the Cypriot grape accession was infected by a closterovirus different from those it had been assayed for. Investigations were therefore conducted for the biological and partial molecular characterization of this virus isolate, which was denoted GLRaV-Cyp1.

For mechanical transmission trials, young leaves from the Cypriot grapevine accession were ground in a mortar in the presence of 0.05 M phosphate buffer pH 7.2, containing 2.5% nicotine and the extract was rubbed on the leaves of glasshouse-grown plants of *Chenopodium amaranticolor*, *C. quinoa, Nicotiana benthamiana, N. cavicola, N. occidentalis, Gomphrena globosa* and *Cucumis sativus*. None of the indicators showed symptoms, no virus was recovered by back inoculation to *Nicotiana* spp. seedlings, and no amplification was obtained when they were PCR-tested using HSP70h-specific primers, as specified below.

In March 2007, GLRaV-Cyp1-infected sources were chip-bud grafted onto a group of five rooted cuttings of healthy cv. Cabernet sauvignon. Comparable cv. Cabernet sauvignon rooted plants were chip-bud grafted with material from vines infected with GLRaV-1 and GLRaV-3 as positive controls. Graft-inoculated plants and non inoculated controls were maintained in a glasshouse at about 24°C. Mild leafroll symptoms appeared on all GLRaV-Cyp1-inoculated plants in September 2007 compared to those inoculated with GLRaV-1 and GLRaV-3, confirming field observations and fulfilling part of the Koch's postulates.

In mealybug transmission trials, *Planococcus ficus* individuals were reared on potato sprouts for several generations under controlled conditions (glasshouse at 26-28°C). Potted GLRaV-Cyp1-infected vines were used as virus source, whereas six healthy potted vines of cv. Cabernet sauvignon were used as recipients. Trials were carried in a glasshouse inside insect-proof cages. For virus acquisition, *P. ficus* crawlers were allowed to move to infected vines by placing infested potato leaves on GLRaV-Cyp1-infected vines. After two weeks healthy Cabernet sauvignon rootlings were placed in the cage in close contact with GLRaV-Cyp1-infected sources infested by mealybugs and the transfer of *P. ficus* crawlers to healthy vines was monitored. Inoculated plants were tested by RT-PCR for the presence of the virus using GLRaV-Cyp1-HSP70h specific primers, 2 weeks post inoculation (Tab. 1). The test was continued on a weekly basis up to eight weeks. RT-PCR tests of 6 to 8 weeks post-inoculation showed a product of the expected size (260 bp) from all vines exposed to viruliferous mealybugs (Fig. 2). This was retained as evidence of the positive transmission of GLRaV-Cyp1 by *P. ficus*. No visible symptoms were observed after 8 weeks.

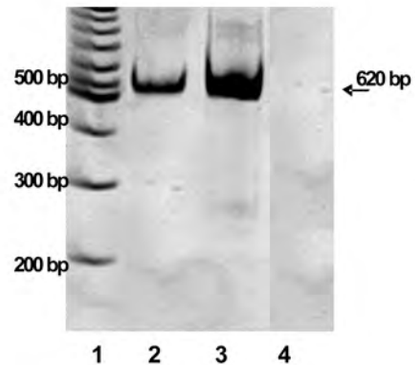

Fig. 1. Amplification of *ca* 620 bp fragment from GLRaV-Cyp1 (lane 2) and GLRaV-3- (lane 3) infected grapevine extracts using degenerate primers (Tian *et al.*, 1996). Negative control of a healthy grapevine (lane 4). DNA Marker is in lane 1.

Table 1. Primers used for PCR amplification of sequence fragments of three GLRaV Cyp-1 genes and relative cycling conditions.

Primers and their sequences (5' → 3')	Gene	Amplicon size (bp)	PCR cycling conditions	
GLRaV-Cyp1-HSP70 (sense) GGACTGGGTGCTCTATTGGA	HSP70	260	58°C, 30 sec 72°C, 30 sec	}35 cycles
GLRaV-Cyp1-HSP70 (antisense) AGAGAAACTTGCGTGCCTGT			1.0 mM $MgCl_2$	
Amp 55-F3 (sense) GGYGGTTCRTTYGCHAARTGGAA	CP	1500	48°C, 1 min 72°C, 1 min	}40 cycles
P23 CP Amp (antisense) TGACATGGWTTCAGATAGAGAG	p23		2.0 mM $MgCl_2$	
GLRaV-Cyp1-CP (sense) GACTGCTAGCAATCCGCCCG	CP	207	58°C, 30 sec 72°C, 30 sec	}35 cycles
GLRaV-Cyp1-CP (antisense) AGTTGTCAGGTTCAGTACGGTG			1.0 mM $MgCl_2$	

Partially purified GLRaV-Cyp1 preparations were obtained from cortical scrapings of mature canes following the procedure described by Namba *et al.* (1991). Electron micrograph from this preparation showed one type of filamentous flexuous particles with distinct cross banding (not shown). Particle fragmentation impaired estimation of their length.

The molecular weight of the coat protein (CP) was determined by boiling concentrated virus preparations for 5 min in the presence of an equal volume of Laemmli (1970) 2x loading buffer (0.125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercapthoethanol and 0.02% bromophenol blue) followed by polyacrylamide gel electrophoresis (SDS-PAGE) at 80V for 30 min and 120 V for 2 h. The gel was stained according to Berger *et al.* (1989). A single band of *ca.* 35 kDa in size was obtained, which is in line with the molecular weight expected for ampelovirus CP subunits (Martelli *et al.*, 2005).

Total nucleic acids (TNAs) extracts were used as template for the partial molecular characterization of GLRaV-Cyp1. In this experiment, TNAs were extracted from approximately 100 mg of cortical scrapings, homogenized in 1 ml grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5.0 and 2.5% w/v PVP-40), and purified with a silica-capture procedure according to Foissac *et al.* (2001).

For cDNA synthesis, degenerate primers designed to amplify a 620 base pair (bp) fragment from the HSP70h gene (Tian *et al.*, 1996) were used in RT-PCR. cDNA was synthesized by mixing 10 µl of TNAs with 1 µl random primers (0.5 µg/µl) and 1.5 µl RNase-free water. The mixture was denatured by boiling at 95°C for 5 min, followed by fast cooling, then mixing with 4 µl M-MLV (5x) 1st strand buffer, 2 µl DTT (0.1 M), 0.5 µl dNTPs (10 mM), 1 µl M-MLV RT enzyme (200 U/µl; Promega, USA). The preparation was incubated at 39°C for 60 min, 70°C for 10 min and finally at 4°C before use or stored at -20°C.

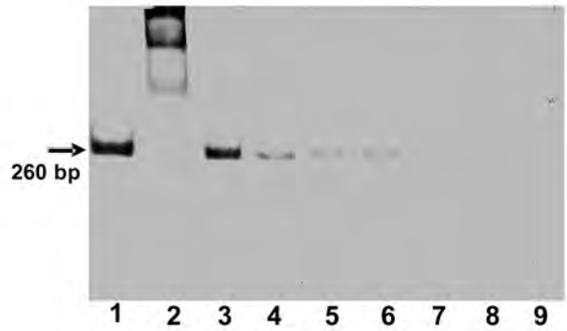

Fig. 2. RT-PCR detection of GLRaV-Cyp1 in cv. Cabernet sauvignon rooted cuttings exposed to presumably viruliferous *P. ficus* (lanes 3 to 8) using virus-specific primers (Table 1). Positive control is in lane 1, marker in lane 2, negative control in lane 9.

The sequence between the two conserved HSP70h-motifs (Tian *et al.*, 1996) was used for designing a set of GLRaV-Cyp1-specific primers (Tab. 1), whose selective performance was proven by the contemporary PCR testing of GLRaV-Cyp1 positive control and other GLRaVs (GLRaV-1, -2, -3, -4, -5,- 6 and -7) isolates. A second set of degenerate primers (Amp 55-F3 sense and P23 CP Amp antisense) for the amplification of GLRaV-Cyp1 major coat protein (CP) and p23 was designed, based on nucleotide alignments of CP/p23

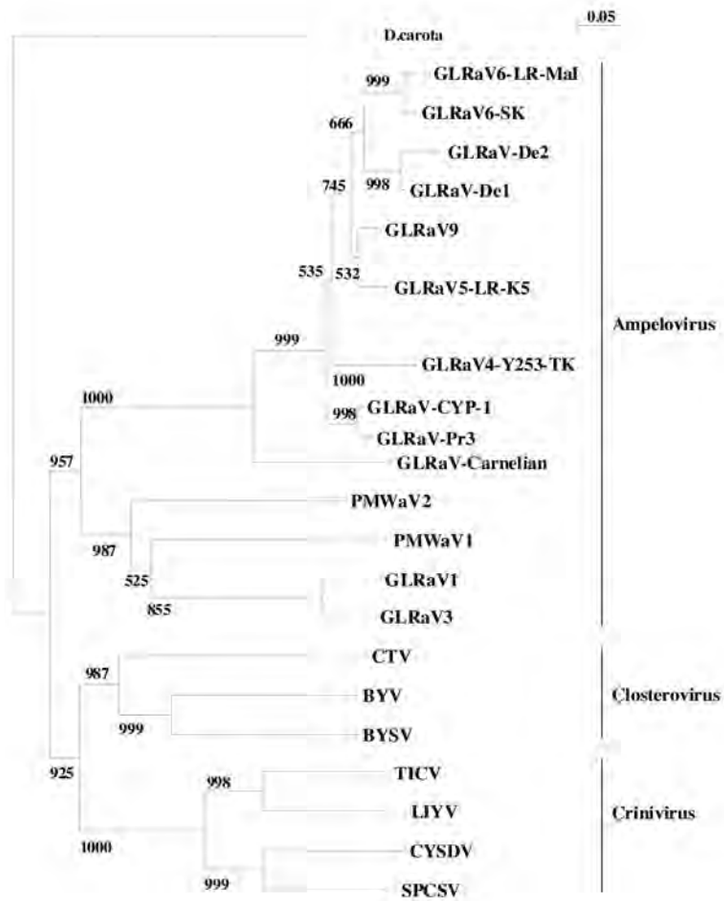

Fig. 3. Phylogenetic tree constructed with HSP70h sequences of different species of the family *Closteroviridae,* including Grapevine leafroll-associated virus (GLRaV) species and isolates. GLRaV-6-LR-MaI (AM745345); GLRaV-6-SK (AM745346); GLRaV-De2* (AM745349); GLRaV-De1* (AM494935); GLRaV-9 (AY297819); GLRaV-5-LR-Pot (AM745344); GLRaV-Pr3 (AM182328); GLRaV-Cyp-1 (FM244689); GLRaV-4-Y253-TK (AM162280); GLRaV-1 (AF195822); GLRaV-3 (NC004667); GLRaV-Carnelian (unpublished sequence- S. Sabanadzovic, personal communication); *Pineapple mealybug wilt-associated virus 2,* PMaWV-2 (AF283103); *Citrus tristeza virus,* CTV (U16304); *Beet yellows virus,* BYV (AAF14302); *Beet yellow stunt virus,* BYSV (U51931); *Tomato infectious chlorosis virus,* TICV (U67449); *Lettuce infectious yellows virus,* LIYV (U15441); *Cucumber yellow stunting disorder virus*, CYSDV (AJ223619); *Sweet potato chlorotic stunt virus*, SPCSV (AJ428555); *Daucus carota* HSP70h (X53852) was used as outgroup. (* Maliogka *et al.*, 2008)

genes of GLRaV-4,-5,-6,-9 and selection of the most conserved nucleotide sequences (Tab. 1). A third set of specific primers (GLRaV-Cyp1-CP-sense and GLRaV-Cyp1 antisense) was designed based on the CP-Cyp1 sequence. This last set of primers was used for detecting Cyp1 in mealybug- infested plants (Tab. 1).

PCR amplicons were electrophoresed in 6% polyacrylamide slab gels and/or ligated directly into pGEM-T Easy (Promega, USA) according to the manufacturer's instructions, and used to transform *Escherichia coli* DH5α cells. DNA from clones was subjected to automated sequencing (Primm, Italy). Sequences were deposited in GenBank under the following accession numbers: FM244689 (HSP70h), FM244690 (CP) and FM244691 (p23).

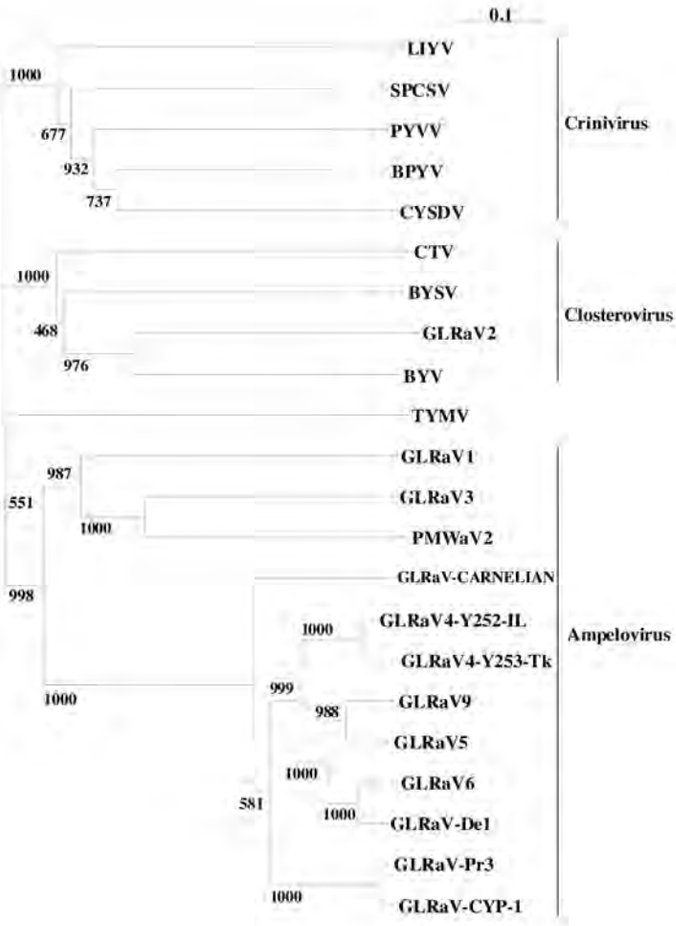

Fig. 4. Phylogenetic tree constructed with CP/CPm sequences of different species of the family *Closteroviridae,* including GLRaV species and isolates. *Sweet potato chlorotic stunt virus,* SPCSV (AJ428555); *Potato yellow vein virus,* PYVV (AJ557129); *Beet pseudo yellow virus,* BPYV (NC005210); *Cucurbit yellow stunting disorder virus,* CYSDV (AJ223619); *Lettuce infectious yellows virus,* LIYV (U15441); *Citrus tristeza virus,* CTV (U16304); *Beet yellow stunt virus,* BYSV (U51931); *Beet yellows virus,* BYV (AAF14302); *Pineapple mealybug wilt-associated virus* 2, PMWaV-2 (AF283103); *Grapevine leafroll-associated virus 1,* GLRaV-1 (AF195822); GLRaV-2 (NC007448); GLRaV-3 (NC004667); GLRaV-4-Y253-TK (AM162279); GLRaV-5 (AF233934); GLRaV-6 (JF467504); GLRaV-9 (AY297819); GLRaV-De1 (AM494935); GLRaV-Pr3 (AM182328); GLRaV-Carnelian (unpublished sequence - S. Sabanadzovic, personal communication); GLRaV-Cyp-1 (FM244690, FM244691); *Turnip yellow mosaic virus,* TYMV (NC004063) was used as outgroup.

Nucleotide and protein sequences were assembled using the Strider 1.1 Program (Marck, 1988). Protein sequences were aligned with the Clustal X program. Homology with other known proteins from the protein information resources (PIR, release 47.0) was determined with the FASTA (Pearson and Lipman, 1988) and BLASTA (Altschul *et al.,* 1990) programs. Phylogenetic trees were constructed and bootstrap analysis made with the programs of the PHYLIP package (Felsenstein, 1989).

In the phylogenetic tree constructed with HSP70h and CP/p23 sequences of several members of the family *Closteroviridae* the ampelovirus clade showed two main sub-clades, one of which comprised GLRaV-1, GLRaV-3 and *Pineapple mealybug wilt-associated* virus 2 (PMWaV-2) and the other the "type strains" of GLRaV-4, -5, -6, -9 plus a number of molecularly divergent isolates of these viruses (Fig. 3 and 4). The structure of both dendrograms was very similar to that of the trees reported by Saldarelli *et al.* (2006) and Maliogka *et al.* (2008), which comprised many of the sequences used in the present study, except for those of GLRaV-Cyp1. In particular, the two sub clades of our trees had a phylogeny repartition comparable to that of the two subgroups of sequences identified by Maliogka *et al.* (2008).

Interestingly, GLRaV-Cyp1 and the Greek isolate GLRaV-Pr3 (Maliogka *et al.*, 2008) grouped together in both trees (Fig. 3 and 4) and showed 94% identity at the amino acid level in the HSP70h and CP/p23 genes (Table 2), thus appearing to be two isolates of the same species. Likewise, a basic similarity could be inferred between GLRaV-5 and GLRaV-9, because of their close clustering in both trees and of the amino acid identity of 92% and 89% in the HSP70h and CP/p23 genes, respectively (Table 2).

Amino acid sequence identity of GLRaV-Cyp1 was 86% or lower with the HSP70h gene and 70% with the CP and p23 genes when compared to other GLRaVs included in the comparison (Table 2). These values are higher than the 10% threshold in the amino acid sequence of relevant gene products (CP, CPm and HSP70h) suggested by the International Committee on Taxonomy of Viruses (ICTV) as one of the relevant species demarcation criteria in the genus *Ampelovirus* (Martelli *et al.*, 2005). In addition to differences in amino acid sequence, the lack of serological relationships between GLRaV-Cyp1 and several other GLRaVs would have suggested that GLRaV-Cyp1 is a new viral species in the genus *Ampelovirus*, which would agree

Table 2. Percent identity at the amino acid level of HSP70h and CP-P23 (bold) gene sequences of different grapevine leafroll-associated viruses.

Virus-isolate	GLRaV-Cyp-1	GLRaV-Pr3*	GLRaV-5	GLRaV-9	GLRaV-De1*	GLRaV-6	GLRaV-4
GLRaV-Cyp-1	0 **0**	94 **94**	86 **68**	84 **70**	85 **51**	83 **68**	85 **69**
GLRaV-Pr3		0 **0**	87 **70**	82 **70**	86 **53**	83 **67**	83 **69**
GLRaV-5			0 **0**	92 **89**	88 **62**	86 **52**	91 **76**
GLRaV-9				0 **0**	85 **62**	85 **83**	86 **74**
GLRaV-De1					0 **0**	89 **65**	86 **56**
GLRaV-6						0 **0**	82 **76**
GLRaV-4							0 **0**

Sequences are the same used for constructing phylogenetic trees.

* Maliogka *et al.* (2008).

with Maliogka *et al.* (2008) suggestion for GLRaV-Pr, a closely related virus to GLRaV-Cyp1. However, this may not be true because serological relationship using available antisera to GLRaVs are not full proof (Saldarelli *et al.*, 2006). In addition, the validity of the 10% difference in the amino acid sequences of the relevant genes suggested by the ICTV as species demarcation criterion needs to be reviewed and re-evaluated.

For example, at least four different molecular variants of *Grapevine leafroll-associated virus 2* (GLRaV-2) have been identified which share from 72 to 80% sequence identity among each other (Angelini *et al.*, 2004; Meng *et al.*, 2005; Bertazzon *et al.*, 2006; Beuve *et al.*, 2007). Thus, in the GLRaV-2 family there is a continuum of molecular variants which do not meet the ICTV species demarcation criterion. This situation resembles that of the ampelovirus clade comprising GLRaV-4, -5, -6 and -9 and a number of their divergent variants (see also Maliogka *et al.*, 2008). One wonders then, whether all the above ampeloviruses may just represent variants of a single species, rather than a bunch of different taxonomic entities, a likelihood in agreement with the results of a current study (N. Abou Ghanem-Sabanadzovic, S. Sabanadzovic and A. Rowhani, personal communication).

GLRaV-Cyp1 appears to occur in countries other than Cyprus. When 121 grapevine accessions from different Mediterranean countries (Albania, Greece, Italy, Jordan, Lebanon, Malta, Palestine, Tunisia and Serbia) present in the grapevine collection of IAMB were tested by RT-PCR, the virus was detected in three vines from Palestine (cvs Beitoni, Sultanina and Zaini), and two from Jordan (cvs Khalili and Abiad).

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