

## PARTIAL CHARACTERIZATION OF TWO DIVERGENT VARIANTS OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 4

P. Saldarelli<sup>1</sup>, P. Cornuet<sup>2</sup>, E. Vigne<sup>2</sup>, F. Talas<sup>4</sup>, I. Bronnenkant<sup>2</sup>, A.M. Dridi<sup>4</sup>, P. Andret-Link<sup>2</sup>, D. Boscia<sup>1</sup>, P. Gugerli<sup>3</sup>, M. Fuchs<sup>2\*</sup> and G.P. Martelli<sup>1</sup>

<sup>1</sup>Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy

<sup>2</sup>Institut National de la Recherche Agronomique, Unité Mixte de Recherche 'Santé de la Vigne et Qualité des Vins', INRA-Université Louis Pasteur, Laboratoire de Virologie, 28 Rue de Herrlisheim, 68021 Colmar, France

<sup>3</sup>Agroscope, Federal Agricultural Research Station of Changins, P.O. Box 254, 1260 Nyon 1, Switzerland

<sup>4</sup>Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010, Valenzano (Bari), Italy

### SUMMARY

Two non mechanically transmissible viruses with filamentous closterovirus-like particles were extracted and partially characterized from leafroll-affected grapevine accessions of cv Koussan from Turkey (Y253) and cv Koudsi from Israel (Y252), both from the reference collection of grapevine viruses and virus-like diseases at the Institut National de la Recherche Agronomique (INRA) in Colmar, France. These viruses, denoted Y253-TK and Y252-IL, were independently investigated in France (INRA-Colmar) and Italy (DPPMA). Y253-TK did not react in DAS-ELISA or RT-PCR assays that would have detected any of the Grapevine leafroll-associated viruses (GLRaVs) described so far; Y252-IL did give weak and inconsistent positive reactions in IEM only with an antiserum to strain LR106 of Grapevine leafroll-associated virus 4 (GLRaV-4). A polyclonal antiserum to isolate Y252-IL decorated the homologous particles and, those of Y253-TK and, at lower dilution, particles of isolate GLRaV-4 LR106. A segment of the heat shock protein p70 homologue (HSP70h) gene, was amplified by RT-PCR of RNA from denatured purified particles of Y253-TK by using a set of degenerate primers. The 183 amino acid polypeptide deduced from the 549 bp HSP70h gene fragment showed a high degree of identity with the cognate genes of GLRaV-4 (95%), GLRaV-5 (91%), GLRaV-6 (84%), and GLRaV-9 (90%), but low identity with those of other GLRaVs (33-35%). The HSP70h of isolates Y252-IL and LR106 showed 95% amino acid identity. The coat protein sequence of isolates Y253-TK showed 99 and 94% amino acid identity with those of isolates Y252-IL and LR106, respectively, with changes concentrated in the N-terminus, a feature that might have a bearing on the reaction with their heterologous antisera. Based on serological and molecular data, it was concluded that

Y253-TK and Y252-IL are distinct, though similar, isolates of GLRaV-4. In two independent surveys in which 110 and 320 grapevine accessions from germplasm collections at Colmar and Bari, respectively, were examined by DAS-ELISA, the antisera As-Y253-TK and As-Y252-IL cross-reacted with the homologous and heterologous viruses.

Whereas As-Y253-TK did not react with any of the other accessions from the French collection tested, As-Y252-IL recognized a virus present in a total of eight grapevine accessions from the Mediterranean area, grown in the Italian germplasm collection.

*Key words:* *Ampelovirus*, GLRaV-4, ELISA, ISEM, Western blot, HSP70h, sequencing, *Closteroviridae*.

### INTRODUCTION

Leafroll, one of the most important virus diseases of grapevines, elicits a pronounced downward rolling and discoloration of the leaves of European grapes, causes significant yield losses, and affects fruit quality (Goheen, 1990; Martelli, 1993; Cabaleiro *et al.*, 1999; Manini and Credi, 2000; Gugerli, 2003).

Nine distinct viruses denoted Grapevine leafroll-associated viruses (GLRaV-1 to -9) have been identified in leafroll-diseased vines (Martelli *et al.*, 2002; Gugerli, 2003). All these viruses spread with infected propagative material but four of them (GLRaV-1, -3, -5, and -9) are naturally transmitted by coccid and pseudococcid vectors (Gugerli, 2003; Sim *et al.*, 2003).

GLRaVs belong to the family *Closteroviridae*, which comprises three genera *Closterovirus*, *Crinivirus* and *Ampelovirus* (Martelli *et al.*, 2002). GLRaV-1, -3, -5, and -9 are classified in the genus *Ampelovirus* of which GLRaV-4, -6, and -8 are tentative members. All GLRaVs are serologically unrelated, except that GLRaV-1 and GLRaV-3 show a very distant relationship, in assays using monoclonal antibodies (Seddas *et al.*, 2000; Gugerli, 2003).

The reference collection of grapevine viruses and virus-like diseases at the Institut National de la Recherche Agronomique (INRA) in Colmar, France has a number of leafroll-diseased accessions, some of which

Corresponding author: P. Saldarelli  
Fax: +39.080.5442911  
E-mail: p.saldarelli@ba.ivv.cnr.it

\* Present address: Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA.

are infected by GLRaV-1, -2, -3, -4, -5, and/or -7 (Greif and Walter, 1997). This collection comprises one accession of cv Koussan from Turkey (Y253) and one accession of cv Koulsi from Israel (Y252), both of which show leafroll symptoms and contain an unidentified isolate of a closterovirus-like virus that was being independently investigated at INRA-Colmar (Y253) or in the Department of Plant Protection and Applied Microbiology (DPPMA) of the University of Bari, Italy (Y252).

Following exchange of information on the work in progress in the two above institutions, it was found that the virus isolates (denoted Y252-IL and Y253-TK) present in the relative grapevine accessions, were very similar and were serologically related. Studies were therefore continued in parallel in the two laboratories and their outcome is reported in the present paper.

## MATERIALS AND METHODS

**Sanitary status of virus sources.** The sanitary status of accessions Y252 and Y253 was assessed by: (i) green-graft inoculation (Walter *et al.*, 1990) of a standard series of *Vitis* indicators (Martelli, 1993); (ii) ELISA testing for the presence of GLRaV-1 to -7 and -9, *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Tomato black ring virus* (TBRV), *Raspberry ringspot*

*virus* (RpRSV), *Grapevine fleck virus* (GFkV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), and *Strawberry latent ringspot virus* (SLRSV) with reagents produced at INRA-Colmar or kindly provided by Dr. D. Gonsalves (Cornell University, Geneva, NY, USA) (As-CA4); (iii) RT-PCR using primers designed on the sequence of the minor capsid protein of GLRaV-8 (GenBank accession number AF233936) and primers specific to the heat shock protein p70 homologue (HSP70h) gene of GLRaV-9 (Alkowni *et al.*, 2004).

Accession Y252 was checked at DPPMA by ELISA with available commercial kits or locally produced reagents for the presence of GVA, GVB, GFLV, GFkV, and all GLRaVs, except for GLRaV-8. For GLRaV-4, an antiserum was used to LR106 (As-CA4), the type strain of this virus, kindly supplied by Dr. J. Hu (University of Hawaii, Honolulu). Both As-CA4 antibodies were sub-stocks originating from the same source.

**Mechanical transmission.** Mechanical transmissions to *Nicotiana benthamiana* and *N. occidentalis* were attempted with crude extracts of infected leaves from field- or greenhouse-grown plants, and concentrated partially purified virus preparations of Y253-TK or Y252-IL.

**Virus purification and antisera production.** Virus particles of Y253-TK were purified from *ca.* 200 g of

**Table 1.** Serological analysis of several leafroll-infected grapevine accessions by DAS-ELISA carried out at INRA-Colmar with Y253-specific immunoglobulins<sup>a</sup>.

| GLRaVs  | Isolate designation | Grapevine variety  | Absorbance at 405nm      |
|---------|---------------------|--------------------|--------------------------|
| 1       | Pn70                | Pinot noir         | 0.195 <sup>c</sup>       |
| 1       | Y233                | Houedi             | 0.184 <sup>c</sup>       |
| 2       | Y206                | Chaouch rose       | 0.180 <sup>c</sup>       |
| 2       | Z122                | Bourboulenc        | 0.217 <sup>c</sup>       |
| 3       | Y285                | Raziki             | 0.205 <sup>c</sup>       |
| 3       | Y163                | Ahmed Sal          | 0.191 <sup>c</sup>       |
| 4       | 6521                | Gamay              | 0.245                    |
| 5       | Y217                | White Emperor      | 0.212 <sup>c</sup>       |
| 6       | 7126                | Chasselas          | 0.240                    |
| 6       | 6504                | Gamay              | 0.259                    |
| 7       | Y243                | Kandari            | 0.221 <sup>c</sup>       |
| 7       | Y276                | Otscha Bala        | 0.192 <sup>c</sup>       |
| 9       | SA-125              | Cabernet Sauvignon | 0.153 <sup>b</sup>       |
| nd      | Y253                | Koussan            | <b>1.091<sup>c</sup></b> |
| nd      | Y252                | Koulsi             | <b>0.732<sup>c</sup></b> |
| Healthy | TG64                | Klevener           | 0.208 <sup>c</sup>       |
| na      | Extraction buffer   | na                 | 0.218 <sup>c</sup>       |

<sup>a</sup> Samples were ground in 200 mM Tris-HCl pH 8.2, 140 mM NaCl, 2% PVP 40, and 0.05% Tween 20 at a 1:5 ratio (w/v). Three independent experiments were made with phloem scrapings in November 2001 and December 2002, respectively, and with mature leaves in September 2001. Figures in bold are positive for Y253. GLRaV-8 was not tested because no serological reagents or infected material were available to us.

<sup>b</sup> GLRaV-9 was tested as semi-purified virus preparation diluted 1:50.

<sup>c</sup> Average of three experiments.

na: not applicable; nd: undetermined

mature leaves using conventional methods with two final cycles of sucrose cushion and  $\text{Cs}_2\text{SO}_4$  gradient centrifugation (Zimmermann *et al.*, 1990; Gugerli *et al.*, 1997). Virus-containing bands were collected, dialyzed against 100 mM Tris-HCl buffer pH 8.2 and 10 mM  $\text{MgCl}_2$ , and used to immunize rabbits.

For purification of Y252-IL particles, aliquots of no less than 15 g of cortical scrapings from mature canes were pulverized in liquid nitrogen, suspended in 0.5 M Tris-HCl pH 8.2 containing 4% PVPP, 0.5% bentonite, 0.2% b-mercaptoethanol, 5% Triton X-100, and 0.01M  $\text{MgSO}_4$ , and submitted to alternate cycles of low- (4000 rpm/20 min) and high-speed (116300 g/40 min) centrifugation (Namba *et al.*, 1991). At Agroscope in Changins, Switzerland, another antiserum was raised to an Australian isolate of GLRaV-9 (Cabernet Sauvignon SA-125, kindly provided by Dr. N. Habili, Waite Diagnostics, Glen Osmond, South Australia).

The protocol for antiserum production was virtually the same for Y252-IL and Y253-TK. Antigens were mixed with an equal volume of Freund's complete adjuvant (Y253-TK) or incomplete adjuvant (Y252-IL) for the first subcutaneous injection and were delivered with Freund's incomplete adjuvant in the two subsequent booster injections. Antisera were collected two weeks after the last injection.

Immunoglobulins were purified from the Y253-TK antiserum by the rivanol precipitation method and purified IgGs were labeled with biotin (Zimmermann *et al.*, 1990). IgG purification from Y252-IL antiserum was through protein A-sepharose columns (Lindmark *et al.*, 1983) after pre-absorption with a partially clarified extract from cortical scrapings of a healthy vine.

**Electron microscopy.** Leaf dips or purified virus preparations from both accessions were placed on formvar/carbon-coated grids, rinsed with phosphate buffered saline (150 mM NaCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM KCl) containing 0.05% Tween 20 (PBS-T) (Y253-TK) or with distilled water (Y252-IL) and negatively stained with 2% aqueous uranyl acetate. Samples were observed with a Philips EM208 (Y253-TK) or a Philips Morgagni (Y252-IL) electron microscope. For Y253-TK virion decoration, grids were pre-coated with rabbit anti-GLRaV immunoglobulins or monoclonal antibodies, and rinsed with PBS-T prior to exposure to the appropriate serological reagents. Y252 particles were decorated without previous immunotrapping.

**DAS-ELISA.** At INRA-Colmar, crude extracts from Y253 mature leaves or bark tissue were used in DAS-ELISA (Zimmermann *et al.*, 1990). Microtiter plates were coated with a 1:2000 dilution of purified Y253-TK IgGs, and biotinylated IgGs were used at a 1:5000 dilution after absorption with 5% (v/v) crude extracts from healthy grapevine leaves. Substrate hydrolysis was recorded at

405 nm with a Titertek Multiscan MCC/340 reader. Samples were considered positive if their  $\text{OD}_{405\text{ nm}}$  readings were at least twice those of healthy controls  $\pm 20\%$ . Several accessions from the virus collections at INRA-Colmar and at the Federal Agricultural Research Station of Changins, Switzerland were used as positive controls, and the heat-treated accession TG64 (*V. vinifera* cv Klevener de Heiligenstein) was used as the healthy control (Table 1).

At DPPMA, crude extracts from grapevine bark tissues were tested in DAS-ELISA with IgGs purified from antisera As-CA4 (Hu *et al.*, 1990) and As-Y252-IL raised during this study, both of which had been pre-absorbed with healthy grapevine extracts. Microtiter plates were coated with IgGs of either antiserum at a dilution of 1:500 (As-CA4) and 1:1000 (As-Y252-IL), and trapped antigens were revealed by incubation with alkaline phosphatase-conjugated IgGs diluted 1:250 (As-CA4) and 1:2000 (As-Y252-IL) followed by addition of substrate (p-nitrophenylphosphate).

**Western blot.** To estimate the Mr of Y253-TK CP subunits, purified virus preparations or crude leaf extracts from accession Y253 and other accessions were mixed with an equal volume of denaturation buffer (125 mM Tris-HCl pH 6.8, 10% SDS, 3.6 M  $\beta$ -mercaptoethanol), dissociated by boiling for 5 min, and separated by electrophoresis on 15% SDS-containing polyacrylamide gels. After transfer onto PVDF membranes (Immobilon™-P, Millipore), proteins were incubated with appropriate virus-specific IgGs, alkaline phosphatase conjugated-goat anti-rabbit antibodies diluted 1:3000, and the chemiluminescent CDP-Star® substrate from the Immun-Star™ kit (Biorad, Hercules, CA, USA). Membranes were briefly (5-45 sec) exposed to X-ray films, which were then developed according to the manufacturer's recommendations (Kodak, Rochester, NY, USA). Mr of viral CP subunits were calculated using low range pre-stained standards (Biorad, Hercules, CA, USA) with values ranging from 22 (lysozyme) to 116 kDa (phosphorylase B).

**RT-PCR.** At INRA-Colmar, a segment of the HSP70h gene was characterized by reverse transcription (RT)-polymerase chain reaction (PCR) from denatured (10 min at 70°C) purified Y253-TK virus preparations. RT was carried out at 42°C for 1 h in a final volume of 20  $\mu\text{l}$  containing 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM  $\text{MgCl}_2$ , 0.5 mM spermidine, 10 mM DTT, 1 mM of each dNTP, 20 U RNasin, 500 ng of random primers, and 15 U of RTase from *Avian myeloblastosis virus*. After denaturation at 95°C for 5 min, the volume was adjusted to 50  $\mu\text{l}$  with sterile water. cDNA amplification was carried out by PCR in 50  $\mu\text{l}$  in the presence of 4  $\mu\text{l}$  of the RT reaction, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM

MgCl<sub>2</sub>, 0.2 mM of each dNTP, 2 U of Taq DNA polymerase, and 50 pmoles of reverse (5'-GAAAGTAC-CACCNCCNARRTC-3' with R = A or G and N = A, C, G, or T) and forward (5'-GGTTTCGATTTYGGNAC-NAC-3' with Y = C or T) primers designed using the consensus-degenerate hybrid oligonucleotide strategy (Rose *et al.*, 1998) in the conserved N-terminus phosphate 1 and 2 motifs (Tian *et al.*, 1996; Dovas and Katis, 2003), according to Gomez Talquenca *et al.* (2003). PCR used a 3 min heating step at 94°C followed by 30 cycles of 25 sec melting at 94°C, 50 sec annealing at 43°C, and 75 sec elongation at 72°C with a final extension of 5 min at 72°C. The reaction products were resolved by electrophoresis in 1.5% agarose gels in 90 mM Tris-borate, 2 mM EDTA pH 8.0, and subsequently visualized under UV light after staining with ethidium bromide.

At DPPMA, a similar approach was used for characterizing a segment of the HSP70h gene of Y252-IL and the whole CP genes of Y252-IL and Y253-TK. To this effect, the primers designed by Routh *et al.* (1998) were used for HSP70h, i.e. HSP4A (5'-CTAAACCAGCGCTGTTG-3') and HSP4B (5'-GTGATACCATAACATACCGACC-3'). In addition, two sets of primers, LRS-for (5'-TGARAGRGCBTGGTGTGCTCC-3' with R = A or G and B = C, G, or T) / LRS-rev (5'-CCAGTTTVCCHGABGATATCCC-3' with H = A, C, or T), and LR4Cpint (5'-GAGAGTGACAAGCACCAGGTGC-3') / LR4Cpfin (5'-TCACCTCCTGTTGCCCA-3') were designed on sequences of the GLRaV-4 type strain LR106, which amplified a region spanning across the 3' terminus of the HSP90 analogue gene (LRS-for) and the 5' end of the CP gene (LRS-rev), and the 3' half of the CP gene (LR4Cpint/LR4Cpfin), respectively. Sequences of primers LRS-for and LRS-rev were kindly provided by Drs. S. Sabanadzovic and N. Abou Ghanem-Sabanadzovic (Mississippi State University, Rockville, USA). RT-PCR was carried out on total RNA preparations from cortical scrapings of dormant cuttings, purified according to Foissac *et al.*, (2000). Conditions for RT, PCR and gel analysis were the same used at INRA-Colmar, except that annealing was at 52°C for 40 sec and 45°C for 30 sec for primers LRS-for/LRS-rev and LR4Cpint/LR4Cpfin, respectively.

**Cloning, sequencing, and sequence data analyses.** RT-PCR-amplified DNA fragments of interest were purified at INRA-Colmar with the QIAquick gel extraction kit (Qiagen, Valencia, USA) after electrophoresis in agarose gels, cloned into plasmid pGEM-T Easy (Promega, Madison, USA) according to the manufacturer's recommendations, and transferred into *Escherichia coli* JM109 cells. Recombinant clones with 500-650 bp inserts digested with *Nco*I and *Not*I were sequenced using the ABI Prism Big Dye Terminator kit and an Applied Biosystems 3100 sequencer at the Sequencing Facility of the Institut de Biologie Moléculaire

des Plantes, CNRS, Strasbourg, France. Nucleotide and amino acid sequences were analyzed using the Vector NTI sequence analysis software packages. The program Clustal W was used for alignment of the nucleotide sequences (Thompson *et al.*, 1994) and the distance matrix based on Jukes and Cantor's model was used to estimate nucleotide divergence. The phylogenetic relationships were determined with the neighbor-joining algorithm of the Clustal W package.

At DPPMA, gel-purified amplicons (Qiagen, QIAquick gel extraction kit) were ligated to a pDrive vector (Qiagen, PCR cloning kit) and transferred to *Escherichia coli* Top10 cells. Recombinant clones were identified by PCR-miniprep using the corresponding set of primers and sequences were obtained by automatic sequencing (MWG, Biotech, Ebersberg, Germany). Multiple nucleotide alignments were done using the same software used at INRA-Colmar. Predictive antigenicity analysis of amino acid sequences was made with the Peptidestructure software (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI, USA). A phylogenetic tree was constructed with CP sequences available from databases using the neighbor-joining algorithm of the Clustal W package.

A minimum of three recombinant clones were analyzed by sequencing in both laboratories.

## RESULTS

**Sanitary status of virus sources.** Indexing on woody indicators showed that accessions Y252 and Y253 were both affected by leafroll and vein mosaic, while Y253 was also affected by Kober stem grooving. DAS-ELISA revealed the presence of GVA in Y253 but of none of the other viruses tested (GLRaV-1 to -7, ArMV, GFkV, GFLV, GVB, RpRSV, TBRV, and SLRSV). No amplification was obtained in RT-PCR from leaf extracts or partially purified Y253-TK preparations with primers designed to amplify the sequences of CPm of GLRaV-8 or the HSP70h gene of GLRaV-9.

ELISA testing of accession Y252 gave consistent negative results with antisera to all viruses tested (GLRaV-1, -2, -3, -5, -6, -7, GVA, GVB, GFLV, and GFkV), except for GLRaV-4, which gave erratic and generally very low responses to the antiserum CA-4 (Hu *et al.*, 1990), a behaviour also observed in IEM.

No virus was recovered by mechanical transmission from accession Y252, whereas only GVA was transmitted from accession Y253.

From these preliminary assays, we concluded that accession Y253 hosted an unreported closterovirus-like virus and that accession Y252 was infected by a virus somewhat related with GLRaV-4.

**Electron microscopy.** Electron microscope observa-

tions of partially purified particles of Y253-TK and Y252-IL showed flexuous rod-shaped particles (Fig. 1A, B and C) with a length within the range reported for virus species of the family *Closteroviridae* (Martelli *et al.*, 2002; Gugerli, 2003), i.e. *ca.* 1900 nm for the majority (68%) of the 40 Y253-TK particles measured, and *ca.* 1800 nm for the longest Y252-IL particles observed.

**Serology.** The antiserum to Y253-TK raised at INRA-Colmar decorated homologous virions at a dilution of 1:100 in ISEM (Fig. 1D) but did not decorate purified particles of GLRaV-1 or GLRaV-9 (not shown). Likewise, antibodies to GLRaV-1, -4, -5, -6, and -9 did not decorate Y253-TK particles (not shown). DAS-ELISA with biotinylated antibodies to Y253-TK showed positive reactions only with the homologous accession Y253 and with Y252. No cross reactions occurred with any of the leafroll-associated closteroviruses tested, i.e. GLRaV-1 to -7 and -9 (Table 1), nor with GVA and GVB. DAS-ELISA absorbance values were higher with phloem scrapings than with leaf crude sap (not shown), petioles, or leaf vein extracts. Adsorbing the As-Y253-TK conjugate with healthy leaf extracts substantially reduced the background level so that OD readings of infected plant samples ( $1.541 \pm 0.43$ , no adsorption, vs  $1.035 \pm 0.25$ , adsorption) were more readily discriminated from OD readings of healthy plant samples ( $0.544 \pm 0.12$ , no adsorption, vs  $0.208 \pm 0.10$ , adsorption). The values represent the mean of five different readings after 1 hr substrate addition.

The antiserum to Y252-IL decorated homologous virions at a dilution of 1:100 (Fig. 1E), particles of Y253-TK at a dilution of 1:100 (not shown) and particles of GLRaV-4 LR106 at a dilution of 1:10 (Fig. 1F), but did not recognize GLRaV-1, -2, -3, -5, and -7 in ELISA. This confirmed the erratic serological relationship between Y252 and GLRaV-4 as revealed by the assays for the assessment of the sanitary status of accession Y252. As-Y252-IL gave clear reactions with the homologous antigen (Y252-IL) and with Y253-TK whereas As-CA4 recognized only the homologous antigen (GLRaV-4 LR106) in ELISA (Table 2).

**Table 2.** Comparative analysis of As-Y252-IL and As-CA4 by DAS-ELISA.

| Isolate designation | Absorbance at 405nm <sup>a</sup> |              |
|---------------------|----------------------------------|--------------|
|                     | As-Y252-IL                       | As-CA4       |
| LR106               | 0.132                            | <b>0.800</b> |
| Y253                | <b>0.350</b>                     | 0.106        |
| Y252                | <b>0.839</b>                     | 0.107        |
| Healthy             | 0.060                            | 0.120        |
| Extraction buffer   | 0.044                            | 0.120        |

<sup>a</sup>OD<sub>405 nm</sub> readings were recorded after 1 h substrate addition. Positive reactions are in bold.

**Surveys for Y253-TK and Y252-IL in grapevine collections.** The availability of As-Y253-TK and As-Y252-IL made it possible to conduct surveys by DAS-ELISA in France and Italy.

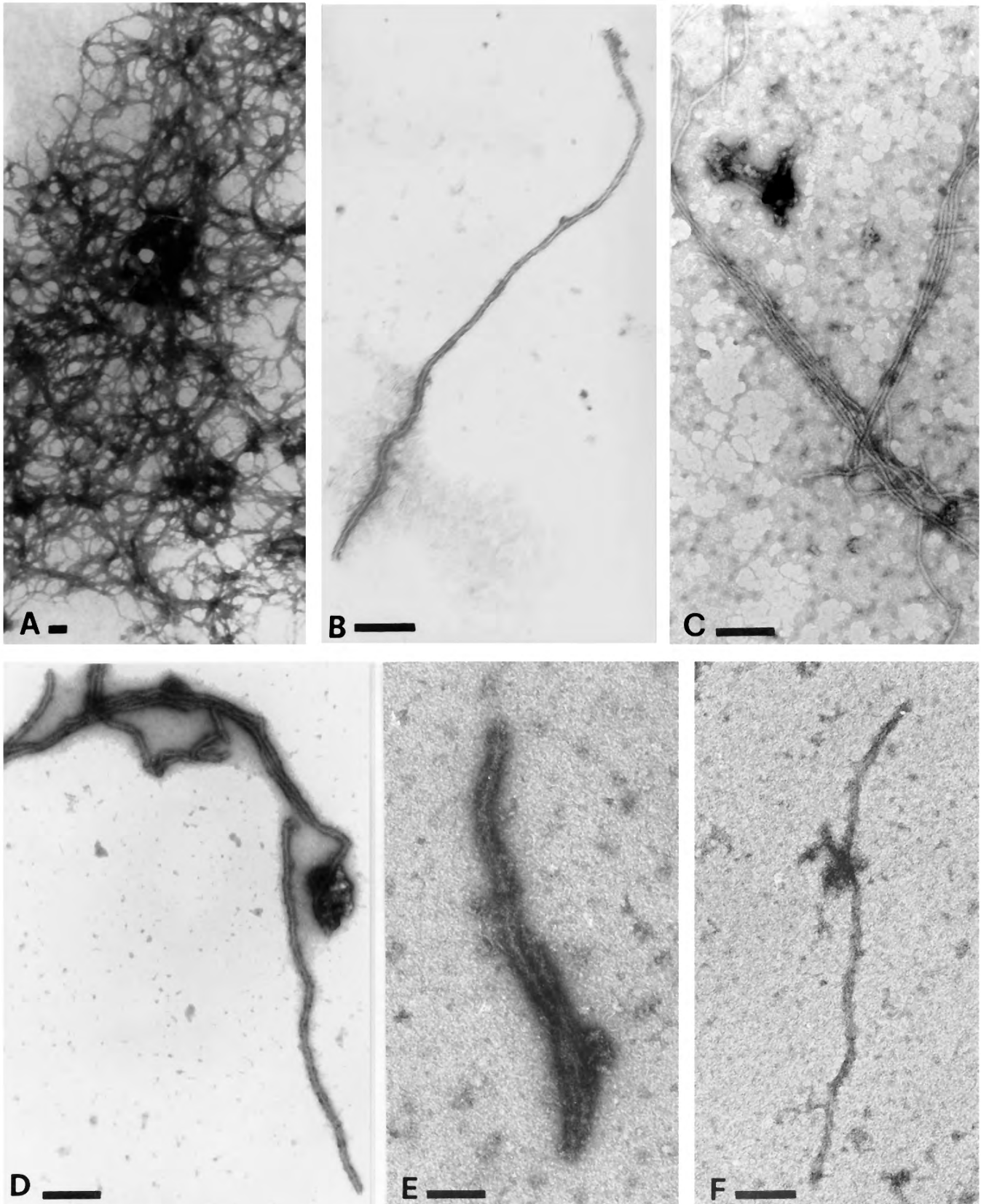
In the INRA reference collection at Colmar, which comprises 110 accessions from 18 different countries (Afghanistan, France, Greece, Hungary, Iran, Israel, Italy, Japan, Lebanon, Russia, Spain, Syria, Switzerland, Tunisia, Turkey, USA, Uzbekistan, and Yemen), As-Y253-TK gave a positive reaction only with accession Y252 (cv Koudsi). It did not recognize an isolate of GLRaV-4 infecting a vine of cv Gamay from Switzerland that is recognized by As-CA4 (Table 1).

In the comparable survey carried out in Italy with both As-Y252-IL and As-CA4 on 320 vines of a collection of the DPPMA comprising accessions from 21 countries (Albania, Afghanistan, Bulgaria, Cyprus, Egypt, former Yugoslavia and USSR, France, Hungary, Israel, Italy, Jordan, Lebanon, Malta, Nigeria, Palestine, People's Republic of China, Spain, Tunisia, Turkey, and USA), As-Y252-IL reacted with the homologous source (Y252) plus seven additional sources, all from Mediterranean countries, including accession Y253 (cv Koussan). None of these sources was recognized by the antiserum As-CA4, which reacted with the homologous source plus nine sources of Mediterranean origin, but different sources from those positive to As-Y252-IL (Table 3). This indicates that there are

**Table 3.** Results of the survey of 320 accessions in the germplasm collection of DPPMA made by DAS-ELISA with antisera As-Y252-IL and As-CA4.

| Accession            | Origin          | ELISA      |        |
|----------------------|-----------------|------------|--------|
|                      |                 | As-Y252-IL | As-CA4 |
| Superior             | Palestine       | -          | +      |
| Cardinal             | Jordan          | -          | +      |
| Rubin                | Italy           | -          | +      |
| Perlette             | Palestine       | -          | +      |
| Dabouqi              | Palestine       | -          | +      |
| Thompson             | Jordan          | -          | +      |
| LR106                | USA             | -          | +      |
| Golden Muscat        | Italy           | -          | +      |
| Superior seedless    | Tunisia         | -          | +      |
| Sant nero            | Tunisia         | -          | +      |
| Abaidi               | Jordan          | +          | -      |
| Abaidi               | Lebanon         | +          | -      |
| Dorable              | Jordan          | +          | -      |
| Biadi                | Jordan          | +          | -      |
| Y253                 | Turkey          | +          | -      |
| Y252                 | Israel          | +          | -      |
| Halawani             | Jordan          | +          | -      |
| Tifaihi              | Lebanon         | +          | -      |
| other 302 accessions | various origins | -          | -      |

Positive and negative reactions are respectively indicated by + and -.

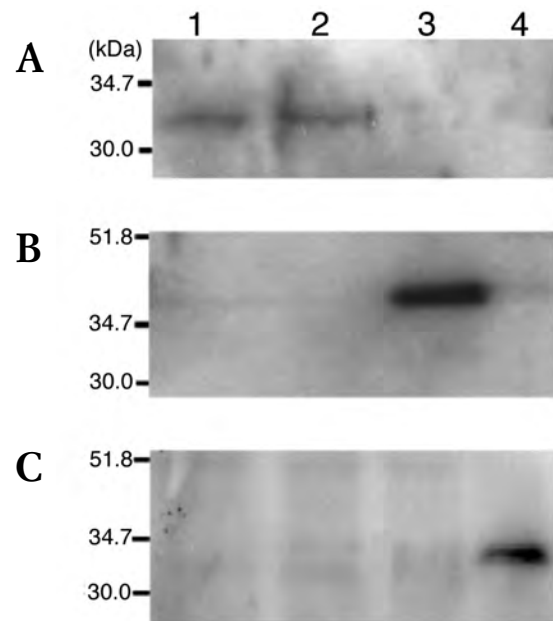


**Fig. 1.** Electronmicrographs of (A) purified Y253-TK particles mounted in uranyl acetate, (B) close-up of a single purified Y253-TK particle, (C) purified Y252-IL particles, (D) Y253-TK and (E) Y252-IL particles heavily decorated by homologous immunoglobulins, and (F) GLRaV-4 LR106 particles decorated with the antiserum to Y252-IL. Scale bar represents 100 nm.

at least two types of serological variants of GLRaV-4 in the Mediterranean area.

**Western blot.** Western blots with the Y253-TK IgGs showed that CP subunits of the homologous virus migrate as a single band with an estimated Mr of *ca.* 32000 (Fig. 2A). This value is identical to that reported for GLRaV-6 (Gugerli *et al.*, 1997) but different from those of other GLRaVs (25, 36, 37, 38, and 43 kDa), including GLRaV-4 (34 kDa) (Martelli *et al.*, 2002; Gugerli, 2003). In addition, no serological relationship was observed between Y253-TK IgGs and GLRaV-1, -2, -4, -6, or -9 CP subunits (not shown). Similarly, monoclonal antibodies to GLRaV-1 (Fig. 2B) and GLRaV-4 from cv Gamay (Fig. 2C) recognized homologous CP subunits but not those of Y253-TK

**Sequencing and sequence analysis.** At INRA-Colmar, an HSP70h amplicon of 549 bp (GenBank accession number DQ325516) was obtained by RT-PCR with degenerate primers from denatured purified Y253-TK preparations. Its sequence showed high degrees of identity at the nucleotide level with the HSP70h genes of GLRaV-4 (91%), -5 (78%), -6 (73%), and -9 (78%), but only 47-49% identity with those of other GLRaVs, and 44-47% with those of other members of the family *Closteroviridae*, except for *Pineapple mealybug wilt-associated virus 1* (PMWaV-1, 64%) and *Plum bark necrosis stem pitting-associated virus* (PBNSPaV, 55%) (Table 4). This sequence encoded a 183 amino acid polypeptide between the HSP70 phosphate motif 1 and 2, that showed a high level of identity with comparable proteins of GLRaV-4 (95%), -5 (91%), -6 (84%), and -9 (90%) but a much lower identity with counterparts of other GLRaVs (33-35%) and other members of the family *Closteroviridae* (28-38%), except for PMWaV-1 (66%) and PBNSPaV (48%) (Table 4).



**Fig. 2.** Western blot analysis of denatured (1) purified Y253-TK CP subunits, (2) leaf crude extract of accession Y253, (3) purified GLRaV-1 CP subunits, and (4) leaf crude extracts of Gamay 6521 infected by GLRaV-4. Proteins were incubated with immunoglobulins to (A) Y253 and (B) GLRaV-1, and with monoclonal antibodies to (C) GLRaV-4.

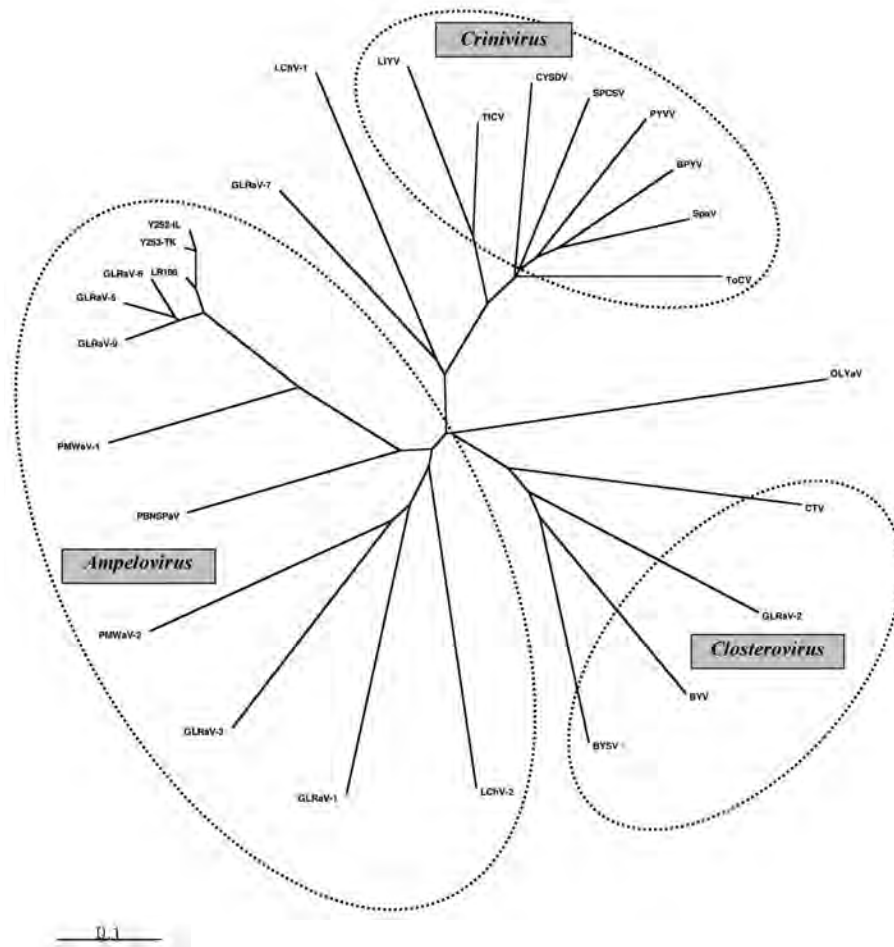
In a phylogenetic tree constructed with HSP70h sequences, Y253-TK and Y252-IL (EMBL accession number AM162280) clustered with GLRaV-4, -5, -6, and -9 in a separate branch clearly distinct from other ampeloviruses (Fig. 3).

At DPPMA, initial attempts to amplify the complete CP genes of Y252-IL and Y253-TK using primers designed on the LR106 CP sequence provided by Dr. A. Rowhani (University of California) were unsuccessful. By contrast, amplification of 492 bp fragments compris-

**Table 4.** Percentage identity between HSP70h and CP coding regions of Y253-TK and other ampelovirus species.

| Virus species   | GenBank accession | Identity (%) |    |    |    |
|---|-------------------|--------------|----|----|----|
|   |                   | HSP70h       |    | CP |    |
|   |                   | nt           | aa | nt | aa |
| GLRaV-1   | AF195822          | 49           | 33 | 46 | 20 |
| GLRaV-3   | AF037268          | 47           | 35 | 46 | 23 |
| GLRaV-4 (LR106)   | AF039553          | 91           | 95 | 90 | 94 |
| GLRaV-4 (Y252)  | AM162280          | 95           | 99 | 95 | 99 |
| GLRaV-5   | AF233934          | 78           | 91 | 74 | 82 |
| GLRaV-6   | AJ496796          | 73           | 84 | na | na |
| GLRaV-9   | AY072797          | 78           | 90 | na | na |
| <i>Little cherry virus-2</i> (LChV-2)                             | AF531505          | 44           | 28 | 42 | 16 |
| <i>Pineapple mealybug-associated virus-1</i> (PMWaV-1)            | AF414119          | 64           | 66 | 57 | 51 |
| <i>Pineapple mealybug-associated virus-2</i> (PMWaV-2)            | AF283103          | 47           | 38 | 45 | 20 |
| <i>Plum bark necrosis stem pitting-associated virus</i> (PBNSPaV) | AF95501           | 55           | 48 | na | na |

na : not applicable



**Fig. 3.** Phylogenetic tree showing relationships among virus species and genera in the family *Closteroviridae* based on HSP70h sequences. The neighbor-joining tree was produced and bootstrapped using CLUSTAL W. Branch lengths are proportional to sequence distances. The scale bar represents a relative genetic distance of 0.1. The GenBank accession numbers are: BPYV (*Beet pseudo-yellows virus*) AY330919; BYSV (*Beet yellow stunt virus*) AAC5562; BYV (*Beet yellow virus*) AAF14302; CTV AAC59627; CYSDV AJ223619; GLRaV-1 (*Grapevine leafroll-associated virus 1*) AF195822; GLRaV-2 (*Grapevine leafroll-associated virus 2*) AF03924; GLRaV-3 (*Grapevine leafroll-associated virus 3*) AF037268; GLRaV-4 (*Grapevine leafroll-associated virus 4*) AF039553; GLRaV-5 (*Grapevine leafroll-associated virus 5*) AF233934; GLRaV-6 (*Grapevine leafroll-associated virus 6*) AJ496796; GLRaV-7 (*Grapevine leafroll-associated virus 7*) Y15897; GLRaV-9 (*Grapevine leafroll-associated virus 9*) AY072797; LChV-1 (*Little cherry virus 1*) Y10237; LChV-2 (*Little cherry virus 2*) AF531505; LIYV (*Lettuce infectious yellows virus*) U15441; OLYaV (*Olive leaf yellowing-associated virus*) AJ440010; PBNPaV (*Plum bark necrosis and stem pitting-associated virus*) AF95501; PMWaV-1 (*Pineapple mealybug wilt-associated virus 1*) AF414119; PMWaV-2 (*Pineapple mealybug wilt-associated virus 2*) AF283103; PYVV (*Potato yellow vein virus*) AJ557129; SPaV (*Strawberry pallidosis associated virus*) AY488138; SPCSV (*Sweet potato chlorotic stunt virus*) AJ428555; TICV (*Tomato infectious chlorosis virus*) AB085602; and ToCV (*Tomato chlorosis virus*) AF024630.

ing about two thirds of the sequence that included the 3' termini of the CP genes of Y252-IL, Y253-TK, and LR106 was successful using primers LR4Cpint/LR4CPfin. These sequenced CP fragments had a homology higher than 99% with one another at the amino acid level.

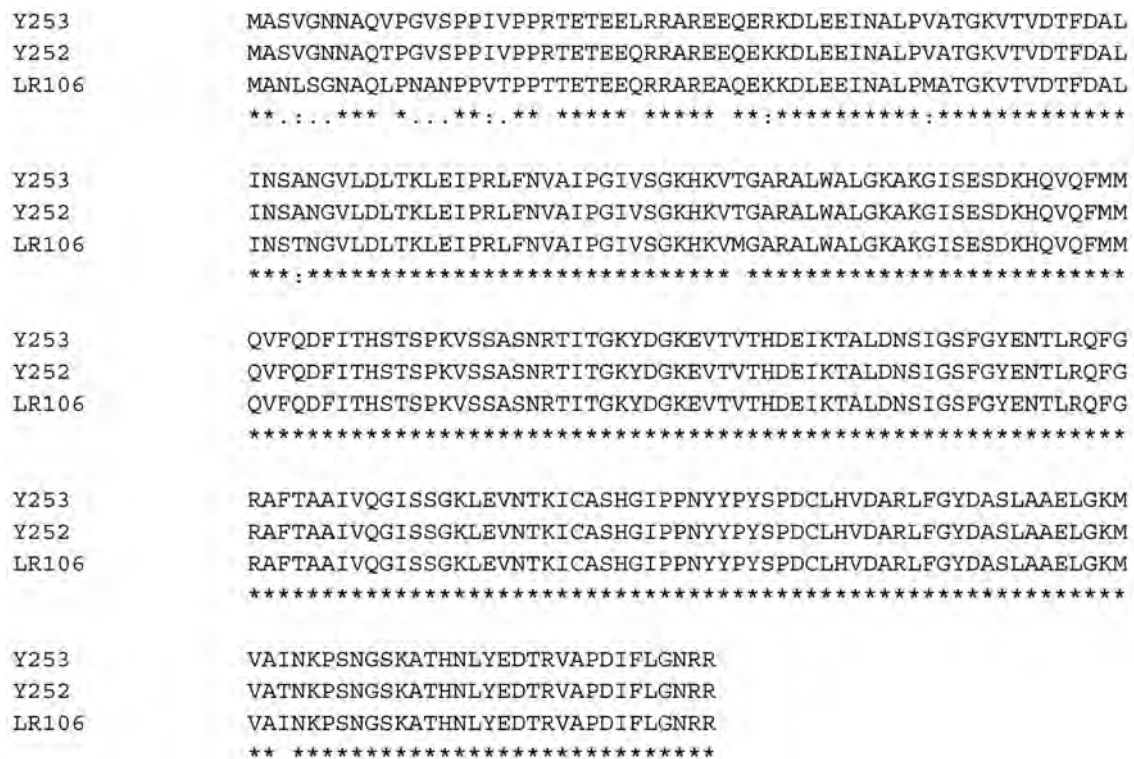
The missing 5' sequence of the CP gene, which included the ATG starting codon, was obtained by amplification with the set of low degeneracy primers LRS-for/LRS-rev. The expected fragment of *ca.* 900 bp was amplified from total RNA extracts of accessions Y252 and Y253. The amplicon from both accessions was cloned and sequenced. The assembled complete CP se-

quences of Y253-TK and Y252-IL (EMBL accession number AM162279 and AM176759) consisted of 272 amino acids with a calculated Mr of 29 520 Da, and differed from the LR106 sequence determined by A. Rowhani (unpublished information) in some residues at the extreme N-terminus (Fig. 4). These localized differences, confirmed by the analysis of three different recombinant plasmids that showed no internal variability, probably explain failures experienced when PCR amplification was attempted using primers located in this region.

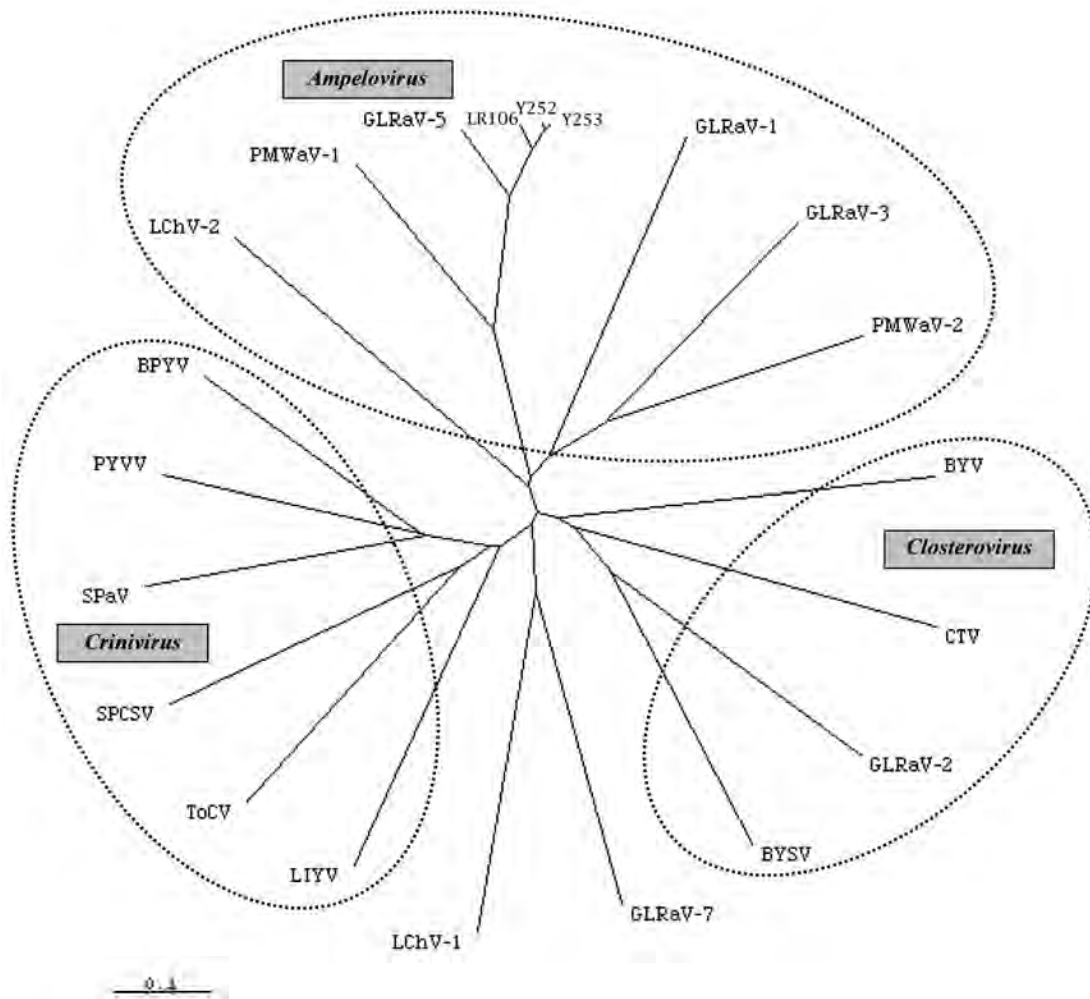
Analysis of putative immunogenic domains in the amino acid sequences of Y253-TK, Y252-IL and LR106 CPs made with the Peptidestructure software showed

**Table 5.** Prediction of antigenic index (AI-Ind) and Surface Probability (SurfPr) values of 25 amino acids (AA) at the extreme N-terminus of the Y253-TK, LR106, and Y252-IL coat proteins by Peptidestructure (differences in antigenic index are highlighted in bold).

| Pos | Y253-TK |        |        | LR106 |        |              | Y252-IL |        |              |
|-----|---------|--------|--------|-------|--------|--------------|---------|--------|--------------|
|     | AA      | SurfPr | AI-Ind | AA    | SurfPr | AI-Ind       | AA      | SurfPr | AI-Ind       |
| 1   | M       | 0.372  | -0.450 | M     | 0.497  | -0.450       | M       | 0.372  | -0.450       |
| 2   | A       | 0.288  | -0.450 | A     | 0.591  | -0.450       | A       | 0.288  | -0.450       |
| 3   | S       | 0.363  | -0.450 | N     | 0.403  | -0.450       | S       | 0.363  | -0.450       |
| 4   | V       | 0.590  | 0.650  | L     | 0.655  | 0.650        | V       | 0.590  | 0.650        |
| 5   | G       | 0.590  | 0.650  | S     | 0.655  | 0.650        | G       | 0.590  | 0.650        |
| 6   | N       | 0.762  | 0.950  | G     | 0.705  | 0.950        | N       | 0.762  | 0.950        |
| 7   | N       | 0.762  | 0.650  | N     | 0.705  | 0.050        | N       | 1.481  | 1.100        |
| 8   | A       | 1.190  | 0.900  | A     | 0.814  | 0.750        | A       | 2.315  | 0.900        |
| 9   | Q       | 0.733  | 0.600  | Q     | 1.323  | 0.750        | Q       | 1.424  | 0.900        |
| 10  | V       | 0.338  | -0.300 | L     | 0.831  | <b>0.600</b> | T       | 0.657  | <b>0.750</b> |
| 11  | P       | 0.448  | -0.450 | P     | 1.323  | <b>1.700</b> | P       | 0.872  | <b>1.250</b> |
| 12  | G       | 0.400  | -0.150 | N     | 1.181  | <b>1.700</b> | G       | 0.779  | <b>1.550</b> |
| 13  | V       | 0.834  | -0.150 | A     | 2.214  | <b>1.700</b> | V       | 0.834  | <b>0.850</b> |
| 14  | S       | 0.378  | -0.150 | N     | 1.063  | <b>1.300</b> | S       | 0.378  | -0.150       |
| 15  | P       | 0.284  | -0.450 | P     | 0.954  | <b>0.950</b> | P       | 0.284  | -0.450       |
| 16  | P       | 0.591  | -0.450 | P     | 1.460  | <b>0.800</b> | P       | 0.591  | -0.450       |
| 17  | I       | 0.682  | -0.300 | V     | 1.404  | <b>0.900</b> | I       | 0.682  | -0.300       |
| 18  | V       | 0.864  | 0.450  | T     | 1.310  | <b>0.900</b> | V       | 0.864  | 0.450        |
| 19  | P       | 0.806  | 0.450  | P     | 1.223  | 0.600        | P       | 0.806  | 0.450        |
| 20  | P       | 1.991  | 1.000  | P     | 2.853  | 1.300        | P       | 1.991  | 1.000        |
| 21  | R       | 3.872  | 1.300  | T     | 2.853  | 1.300        | R       | 3.872  | 1.300        |
| 22  | T       | 4.337  | 0.900  | T     | 3.196  | 0.900        | T       | 4.337  | 0.900        |
| 23  | E       | 4.857  | 0.900  | E     | 3.579  | 0.900        | E       | 4.857  | 0.900        |
| 24  | T       | 2.045  | 0.900  | T     | 4.295  | 0.900        | T       | 4.295  | 0.900        |
| 25  | E       | 2.776  | 0.900  | E     | 5.829  | 0.900        | E       | 5.829  | 0.900        |



**Fig. 4.** Amino acid alignment of the CP sequences of Y253-TK, Y252-IL, and LR106. Note distinct differences at the extreme N-terminus.



**Fig. 5.** Phylogenetic tree showing relationships among virus species and genera in the family *Closteroviridae* based on CP sequences. The neighbor-joining tree was produced and bootstrapped using CLUSTAL W. Branch lengths are proportional to sequence distances. The scale bar represents a relative genetic distance of 0.1. The GenBank accession numbers are: BPYV AAQ97390; BYSV AAC55665; BYV AAA72955.1; GLRaV-1 AF195822; GLRaV-2 CAA74566.1; GLRaV-3 NP\_813801; GLRaV-7 040102; LChV-1 CAA71290; LChV-2 AAM96228; PMWaV-1 AAF14119; PMWaV-2 AAG13943; PYVV CAD89686; SPaV AAS79680; SPCSV CAA56919; and ToCV AAR15080.

that there are distinct differences in the antigenic index of the three polypeptides in a region at the N-terminus (Table 5). Putative epitopes in the LR106 CP sequence are partially or not present in Y252-IL and Y253-TK, respectively. This may explain, at least in part, the inconsistency observed in IEM and ELISA. The evidence that a single mutation at position 10 between Y253-TK and Y252-IL results in a marked change of epitope distribution, is probably explained by the rationale of the Peptidestructure software, which sums several measures of secondary structures to calculate the antigenic index.

Phylogenetic analysis of the Y253-TK and Y252-IL CP genes matched the topology of the HSP70h genes in the phylogenetic tree constructed with CP sequences of members of the family *Closteroviridae* (Fig. 5), supporting the existence of a distinct branch that comprises GLRaV-4, -5, -6, and -9 in a coherent cluster.

## DISCUSSION

Results of the present investigation have shown that Y252-IL and Y253-TK are members of the genus *Ampelovirus*, closely related to one another and more distantly related to GLRaV-4, of which they represent divergent variants. This conclusion conflicts with previous findings suggesting that Y-253-TK could be a separate virus, based primarily on the lack of serological cross reactivity with other grapevine leafroll-associated viruses, including GLRaV-4 (Cornuet *et al.*, 2003).

It has been now ascertained that the HSP70h genes of Y253-TK and Y252-IL have 99% identity at the amino acid level with one another, whereas the identity with the comparable gene of GLRaV-4 LR106 is 95% for both of them. As to CPs, the identity of Y253-TK with GLRaV-4 LR106 and Y252-IL is 94 and 99%, respectively, at the

amino acid level. These molecular data suggest that Y253-TK and Y252-IL are very similar and that both are closely related but distinguishable variants of LR106, the reference strain of GLRaV-4 used in this study.

The serological data matched the molecular data, in that the results of IEM tests (particle decoration) confirm the relationship among the three viruses (Y253-TK, Y252-IL, and LR106) whereas ELISA provided conflicting inconclusive results. Thus, although serological information remains one of the major traits for species discrimination within the genus *Ampelovirus* (Martelli *et al.*, 2005), the determination of the nucleotide sequence of CP and HSP70h genes seems to be required for the establishment of a new GLRaV species.

The serological and molecular variability described here among GLRaV-4 isolates highlights the difficulty in accurately diagnosing the presence of this ampelovirus. More work will be needed to develop a universal laboratory detection test either by mixing several antibodies in DAS-ELISA or by using primers specific for the central or 3', but not 5', end regions of the CP gene in RT-PCR assays.

#### ACKNOWLEDGEMENTS

We are grateful to Dr. Dennis Gonsalves for supplying antiserum to CA-4, Dr. Nuredin Habili for sending GLRaV-9-infected material, and Drs. A. Rowhani (Department of Plant Pathology, University of California, Davis, CA, USA), S. Sabanadzovic and N. Abou Ghanem-Sabanadzovic for providing unpublished information on GLRaV-4 and GLRaV-9. We thank René Legin for graft-indexing, Catherine Reinbold and Prof. M.A. Castellano for assistance with electron microscopy, and Dr. L.M. Yepes for critically reading the manuscript.

#### REFERENCES

- Alkowni R., Rowhani A., Daubert S., Golino D.A., 2004. Partial characterization of a putative new ampelovirus associated with grapevine leafroll disease. *Journal of Plant Pathology* **86**: 123-133.
- Cabaleiro C., Segura A., Garcia-Berrios J.J., 1999. Effects of Grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv Alabarino following contamination in the field. *American Journal of Enology and Viticulture* **50**: 40-44.
- Cornuet P., Andret P., Vigne E., Fuchs M., 2003. Identification and characterization of a putative new ampelovirus species associated to grapevine leafroll. In: *Extended Abstracts of the 14th Meeting of ICVG, Locorotondo, Italy* 2003, 34 (<http://www.agr.uniba.it/ICVG2003>).
- Dovas C.I., Katis N.I., 2003. A spot multiplex nested RT-PCR for the simultaneous and generic detection of viruses involved in the aetiology of grapevine leafroll and rugose wood of grapevine. *Journal of Virological Methods* **109**: 217-226.
- Foissac X., Svanella-Dumas L., Dulucq M.J., Candresse T., Gentil P., 2000. Polyvalent detection of fruit tree tricho, capillo, and foveaviruses by nested RT-PCR using degenerate and inosine containing primers (PDO RT-PCR). *Acta Horticulturae* **550**: 37-44.
- Goheen A.C., 1990. Leafroll. In: R.C. Pearson and A.C. Goheen (eds). *Compendium of grape diseases*, pp. 52. APS Press, St Paul, USA.
- Gomez Talquenca G.S., Gracia O., Garcia Lampasona S.G., Grau O., 2003. A survey for *Closteroviridae* family members in Argentinean vineyards. In: *Extended Abstracts of the 14th Meeting of ICVG, Locorotondo, Italy* 2003: 43 (<http://www.agr.uniba.it/ICVG2003>).
- Greif C., Walter B., 1997. The European reference collection of grapevine virus diseases. In: B. Walter (ed.). *Sanitary selection of the grapevine. Protocols for detection of viruses and virus-like diseases*, pp. 171-181. Institut National de la Recherche Agronomique, Paris, France.
- Gugerli P., 2003. Grapevine leafroll and related viruses. In: *Extended Abstracts of the 14th Meeting of ICVG, Locorotondo, Italy* 2003: 25-31 (<http://www.agr.uniba.it/ICVG2003>).
- Gugerli P., Brugger J.J., Ramel M.E., 1997. Identification immuno-chimique du 6ème virus associé à la maladie de l'enroulement de la vigne et amélioration des techniques de diagnostic pour la sélection sanitaire en viticulture. *Revue Suisse de Viticulture, Arboriculture et Horticulture* **29**: 137-141.
- Hu J.S., Gonsalves D., Teliz D., 1990. Characterization of closterovirus-like particles associated with grapevine leafroll disease. *Journal of Phytopathology* **128**: 1-14.
- Lindmark R., Thorén-Tolling K., Sjöquist J., 1983. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. *Journal of Immunological Methods* **62**: 1-13.
- Mannini F., Credi R., 2000. Appraisal of agronomic and enological modifications in the performances of grapevine clones after virus eradication. In: *Extended Abstracts of the 13th Meeting of ICVG, Adelaide, Australia* 2000, 151-154.
- Martelli G.P., 1993. Graft-transmissible diseases of grapevines. Handbook for detection and diagnosis. FAO Publication Division, Rome, Italy.
- Martelli G.P., Agranowsky A.A., Bar-Joseph M., Boscia D., Candresse T., Coutts R.H.A., Dolja V.V., Falk B.W., Gonsalves D., Jelkmann W., Karasev A.V., Minafra A., Namba S., Vetten H.J., Wisler G.C., Yoshikawa N., 2002. The family *Closteroviridae* revised. *Archives of Virology* **147**: 2039-2044.
- Martelli G.P., Agranowsky A.A., Bar-Joseph M., Boscia D., Candresse T., Coutts R.H.A., Dolja V.V., Falk B.W., Gonsalves D., Jelkmann W., Karasev A.V., Minafra A., Namba S., Vetten H.J., Wisler G.C., Yoshikawa N., 2005. Family *Closteroviridae*. In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (eds.). *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 1077-1087. Elsevier/Academic Press, San Diego, USA.

- Namba S., Boscia D., Azzam O., Maixner M., Hu J.S., Golino D., Gonsalves D., 1991. Purification and properties of closteroviruslike particles associated with grapevine corky bark disease. *Phytopathology* **81**: 964-970.
- Rose T.M., Schultz E.R., Henikoff J.G., Pietrokovski S., McCallum C.M., Henikoff S., 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Research* **26**: 1628-1635.
- Routh G., Zhang Y.P., Saldarelli P., Rowhani A., 1998. Use of degenerate primers for partial sequencing and RT-PCR-based assays of grapevine leafroll-associated viruses 4 and 5. *Phytopathology* **88**: 1238-1243.
- Seddas A., Haidar M., Greif C., Jacquet C., Cloquemin G., Walter B., 2000. Establishment of a relationship between grapevine leafroll closteroviruses 1 and 3 by use of monoclonal antibodies. *Plant Pathology* **49**: 80-85.
- Sim S.T., Rowhani A., Alhowni R., Golino D.A., 2003. Experimental transmission of *Grapevine leafroll associated viruses* 5 and 9 by longtailed mealybugs. In: *Extended Abstracts of the 14th Meeting of ICVG, Locorotondo, Italy 2003*, 211-212 (<http://www.agr.uniba.it/ICVG2003>).
- Thompson J.D., Higgins D.G., Gibson T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.
- Tian T., Klaassen V.A., Soong J., Wisler G., Duffus J.E., Falk B.W., 1996. Generation of cDNAs specific to lettuce infectious yellows closterovirus and other whitely-transmitted viruses by RT-PCR and degenerate oligonucleotide primers corresponding to the closterovirus gene encoding the heat shock protein 70 homolog. *Phytopathology* **86**: 1167-1173.
- Walter B., Bass P., Legin R., Martin C., Vernoy R., Collas A., Vesselle G., 1990. The use of a green-grafting technique for the detection of virus-like diseases of the grapevine. *Journal of Phytopathology* **128**: 137-145.
- Zimmermann D., Bass P., Legin R., Walter B., 1990. Characterization and serological detection of four clostero-like particles associated with leafroll disease on grapevine. *Journal of Phytopathology* **130**: 205-218.

Received 14 December, 2005

Accepted 13 March, 2006