# COMPARISON OF THREE TECHNIQUES FOR DETECTION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1

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**Summary. –** Thirty seven plants of grapevine from the Research Station of Viticulture, Karlštejn was examined for the presence of leafroll viruses. Grapevine leafroll-associated virus 1 (GLRaV-1) was detected in the grapevines plants tested using double-antibody sandwich ELISA (DAS-ELISA), RT-PCR and molecular hybridization with non-radioactive RNA probes. Both molecular methods were based on a detection of the GLRaV-1 heat-shock protein 70 (HSP70) gene and showed a higher sensitivity in the detection of GLRaV-1 compared to DAS-ELISA. RNA probes are considered more suitable for the GLRaV-1 detection, as their application can overcome potential minor sequence variability, which may cause the detection by RT-PCR less reliable, especially when the variability occurs in the genome region targeted by RT-PCR primers. Based on additional DAS-ELISA, a mixed infection of GLRaV-1 and Grapevine leafroll-associated virus 3 (GLRaV-3) occurred frequently, while a mixed infection of GLRaV-1 and Grapevine virus A (GVA) or Grapevine fleck virus (GFkV) or a multiple infection of GLRaV-1, GLRaV-3 and GFkV occurred rarely in the tested plants. A mixed infection of all the four viruses mentioned above was not observed.

**Key words:** Grapevine leafroll-associated virus 1; ampelovirus; HSP 70 gene; RT-PCR; non-radioactive probe; DAS-ELISA

## Introduction

Leafroll is a damaging disease of grapevine causing yield losses of up to 40% (Woodham *et al*., 1984). Several serologically distinct viruses of the *Closteroviridae* family have been associated with this disease (Boscia *et al*., 1995). One of the most important species is the Grapevine leafroll-associated virus 1 (GLRaV-1), a member of the *Ampelovirus* genus (Martelli *et al*., 2002; Habili *et al*., 1996). It has been found in 70% of leafroll-infected grapevines in France (Zimmermann *et al*., 1990) and is widely distributed in a basic propagation material of many grapevine clones in the Czech Republic (Komínek and Holleinová, 2003). The detection of GLRaV-1 is based mainly on the grafting on indicator plants (Rowhani *et al*., 1997), serological tests using polyclonal or monoclonal antibodies (Seddas *et al*., 2000) and PCR (Dovas and Katis, 2003).

Apart from the transmission via vegetative propagation and grafting, GLRaV-1 has been reported to be transmitted by the scale insect *Parthenolecanium corni* and the mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* (Sforza *et al*., 2003).

Filamentous particles of GLRaV-1 contain a positive-sense RNA genome of approximately 19.5 kb and a 39 K coat protein (CP). The genome consists of 10 major ORFs

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**Abbreviations:** dig = digoxigenin; CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; GLRaV-1 = Grapevine leafroll-associated virus 1; GLRaV-2 = Grapevine leafroll-associated virus 2; GLRaV-3 = Grapevine leafroll-associated virus 3; GLRaV-4 = Grapevine leafroll-associated virus 4; GLRaV-5 = Grapevine leafroll-associated virus 5; GLRaV-6 = Grapevine leafroll-associated virus 6; GLRaV-7 = Grapevine leafroll-associated virus 7; GLRaV-8 = Grapevine leafroll-associated virus 8; GVA = Grapevine virus A; GFkV = Grapevine fleck virus; HSP70 = heat-shock protein 70; AP = alkaline phosphatase; SDS = sodium dodecyl sulphate; SSC = saline-sodium citrate buffer; NBT = nitroblue tetrazolium; BCIP = 5-bromo 4-chloro 3-indolyl phosphate

encoding in the 5' to 3' direction a putative helicase, RNA-dependent RNA polymerase, a small hydrophobic protein, a heat shock protein 70 (HSP70) homolog, HSP90-like protein, CP, two diverged CPs (CPd1 and CPd2), and two other proteins of unknown function (Gugerli *et al*., 1984; Fazeli and Rezaian, 2000).

Our work was aimed at improvement of diagnostics of this serious grapevine pathogen in the Czech Republic by comparative evaluation of three different methods.

## Materials and Methods

*Plant material.* Plants of grapevine (*Vitis vinifera* L.), a prebasic propagation material grown at the Research Station of Viticulture Karlštejn, Czech Republic has been planted in 1988. The plants were checked for the presence of GLRaV-1 by visual examination in the second half of the vegetation period for the presence of leafroll symptoms, namely downrolling the leaf blades and premature autumn leaf discoloration. Thirty seven grapevine plants displaying typical leafroll symptoms were selected for further evaluation. They consisted of cv. Müller-Thurgau (20), Pinot Blanc (6), Portugais Bleu (4), Saint Laurent (4), Silvaner (2), and Gewürztraminer (1).

*DAS-ELISA.* The plants were tested by DAS-ELISA for the presence of GLRaV-1 and other leafroll viruses: Grapevine leafroll-associated virus 2 (GLRaV-2), GLRaV-3, Grapevine leafroll-associated virus 5 (GLRaV-5), Grapevine leafroll-associated virus 6 (GLRaV-6), Grapevine leafroll-associated virus 7 (GLRaV-7). Also the presence of GVA and GFkV was searched for, because these viruses were found to be widely spread at the Research Station of Viticulture Karlštejn (Komínek and Holleinová, 2003). The dormant canes were sampled from examined grapevine plants during winter (Rowhani *et al*., 1992). The samples were prepared by grinding 0.3 g of phloem scrapings in 6 ml of an extraction buffer (2.4 g of Tris-HCl, 8.0 g of NaCl, 20.0 g of PVP K25, 0.5 ml of Tween 20, 0.2 g of KCl, 0.2 g of $NaN_3$ per 1 liter, pH adjusted to 7.4). Antisera for GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7, GVA and GFkV were from Agritest (Italy), for GLRaV-5 from Bio-Rad (France) and for GLRaV-6 from Bioreba (Switzerland). The antisera were used according to the instructions of manufacturers. Commercially purchased negative and positive controls to individual viruses as well as a buffer control were included in every plate. To evaluate the suitability of the DAS-ELISA detection method and reproducibility of results the tests for GLRaV-1 were repeated every year in 2001–2003. A grapevine plant was considered positive when at least one test during the three years was positive.

*RNA isolation.* Total RNA was isolated from phloem scrapings from grapevine plants using the Concert™ Plant Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, about 0.1 g of plant tissue was ground in liquid nitrogen. Five ml of the reagent was added and the suspension was mixed and clarified by centrifugation. The resulting supernatant was collected and mixed with 100 µl of 5 M NaCl and 300 µl of chloroform. After shaking and centrifugation the aqueous phase was saved and used for RNA precipitation with isopropyl alcohol. The pelleted RNA was washed with 75% ethanol by centrifugation and dissolved in 30 µl of water. RNA samples were stored at -80°C until assayed by RT-PCR and molecular hybridization.

*Cloning and nucleotide sequencing.* The One-Step RT-PCR Kit (Qiagen) was used for RT-PCR. A nested RT-PCR was used for the amplification of HSP70 gene. The respective primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) on the basis of the only complete sequence of GLRaV-1 (Acc. No AF195822) available in the GenBank (Benson *et al*., 2003). All nucleotide positions mentioned in this article are numbered according to this sequence. The following external primers were designed outside the HSP70 gene: a sense primer 5'-AGGTAC GTAGCTCAACCGTAGA-3' (nt 3684–3705) and an antisense primer 5'-GTTCCTGTGAACCAAACCAACG-3' (nt 5345–5366). The following internal primers were used: a sense primer 5'-GATTTTC GATCCCCACGCAAGA-3' (nt 3755–3774) and an antisense primer 5'-TTAGACGGTCTGTAGTCCGTAC-3' (nt 5025–5046). The expected length of the PCR product was 1291 bp.

The TaKaRa *Taq* Ex polymerase (TaKaRa Bio Europe) was used for the nested PCR. Fragments corresponding to the expected length were gel-purified with the QIAquick® Gel Extraction Kit (Qiagen) and cloned into pGEM®-T Easy plasmid vector (Promega). Recombinant plasmids were propagated in JM109 cells and isolated using the JETquick Plasmid Miniprep Spin Kit (Genomed). Inserts were commercially sequenced with MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences). Two clones were sequenced in both strands using universal pUC primers.

*RT-PCR detection of GLRaV-1.* Use of the sense primer 5'-CAGGCGTCGTTTGTACTGTG-3' (nt 4125–4144), the antisense primer 5'-TCGGACAGCGTTTAAGTTCC-3' (nt 4645–4664) and the One-Step RT-PCR Kit (Qiagen) in an one-step RT-PCR resulted in a product of 540 bp. Briefly, 2 µl of RNA sample was mixed with both primers (0.8 µl each, concentration 100 pmol/µl each), boiled (100°C) for 10 mins, quickly chilled on ice and left to stand at room temperature for 25 mins. Then a 5x Qiagen buffer, dNTP Mixture, Q solution, Qiagen Enzyme Mixture, 40 U of RNaseOUT (Invitrogen) and water to final volume of 50 µl were added. The reaction was performed in a PTC200 type thermocycler (MJ Research). The reverse transcription step consisted of 45°C/60 mins and 95°C/15 mins. The PCR consisted of 40 cycles of 94°C/1 min, 55°C/1 min and 72°C/1 min with final elongation at 72°C for 10 mins. The PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and examined under UV light.

*GLRaV-1 detection by molecular hybridization.* To prepare probes, pGEM®-T Easy plasmids with inserted PCR fragments corresponding to (i) almost entire HSP70 gene and (ii) inner part of the first fragment (Fig. 1) were digested with *Nsi*I (sense probes) or *Apa*I (antisense probes). Linearized plasmids were purified with phenol-chloroform and ethanol precipitated. The DIG-Northern Starter Kit (Roche) was used for digoxigenin-labeling of RNA probes. Ten µl of a purified plasmid was mixed with 4 µl of NTP mixture, 4 µl of 5 x transcription buffer and 2 µl of T7 or SP6 RNA polymerase. After incubation at 42°C for 1 hr, 2 µl of DNase was added and the incubation continued for 15 mins at 37°C. The reaction was terminated by adding EDTA to a final concentration of 0.02 mol/l. The concentration of the obtained probe was estimated by comparison with standards included in the kit by dot blotting, staining with NBT/BCIP and colorimetry. In this way,

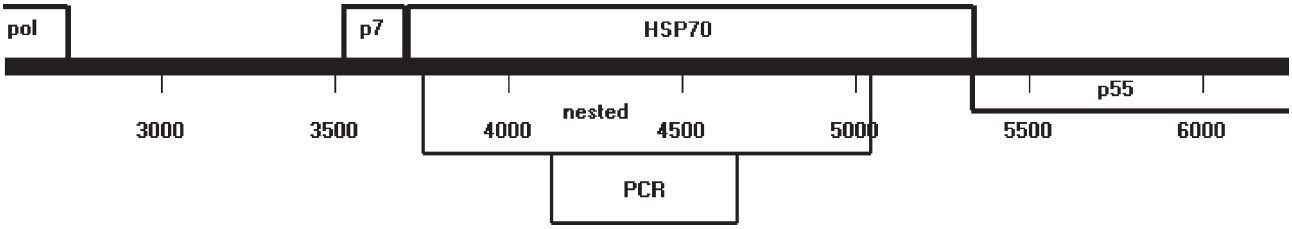


**Fig. 1**

**The GLRaV-1 genome part spanning the HSP70 gene**

Nucleotide numbering corresponds to AF195822. Viral genomic RNA (long solid line). Pol = polymerase gene, p7 = the gene for small hydrophobic protein, HSP70 = HSP70 gene, nested = the region covered by the long hybridization probes and nested RT-PCR primers for sequencing, PCR = the region covered by the short hybridization probes and detection RT-PCR primers, p55 = the 55 K gene protein.

up to 8 µg of digoxigenin-labeled RNA probe was obtained. A dot blot hybridization was used to detect GLRaV-1. RNA samples (1–2 µl) were loaded on Zeta-Probe membranes (Bio-Rad). RNA was fixed to the membrane by irradiation with UV light from a transilluminator for 10 mins.

For the hybridization and subsequent immunological detection of digoxigenin-labeled probes, the DIG Wash and Block Buffer Set (Roche) was used. The blots were pretreated for 30 mins with a prewarmed DIG-Easy Hyb solution at 68°C. Ten ml of DIG-Easy Hyb solution prewarmed to 68°C was mixed with 400 ng of a heat-denatured RNA probe and added to the blot. Hybridization was carried out overnight at 68°C with gentle agitating. The blot was washed with 2 x SSC containing 0.1% SDS for 2 x 5 mins at 25°C, 0.1 x SSC containing 0.1% SDS for 2 x 15 min at 68°C with gentle agitating, and the washing buffer for 5 mins at 25°C. Then, the blot was incubated in a blocking solution for 30 mins at 25°C followed by an antibody solution (Anti-digoxigenin-AP diluted 1:10,000 in a blocking solution). Finally, the blot was washed with a washing buffer for 2 x 15 mins and incubated in a detection buffer for 5 mins followed by a NBT/BCIP (Roche) solution in a detection buffer (staining). After obtaining full color, the reaction was stopped by rinsing the blot under tap water.

## Results

*DAS-ELISA detection of GLRaV-1, GLRaV-3, GVA and GFkV*

Thirty-seven grapevine plants exhibiting leafroll symptoms were screened by DAS-ELISA for the virus presence for three consecutive years (Table 1). Twenty of these plants were found to be positive for GLRaV-1, while 17 were negative. Most of the GLRaV-1-negative plants (16) were found positive for GLRaV-3. Moreover, four plants were found to be infected with GVA and five with GFkV, always in a mixed infection with GLRaV-1 or GLRaV-3. Only four symptomatic plants of the group tested were negative for any virus under screening.

*Characterization of a Czech isolate of GLRaV-1*

A partial sequence (nt 3710–5341, AF195822) of the HSP70 gene of a Czech isolate of GLRaV-1 was determined in order to design primers for the RT-PCR detection of this virus. The RT-PCR with primers designed to amplify whole HSP70 gene failed. Therefore, new primers were designed inside the expected fragment near its ends. This nested RT-PCR turned out to be more suitable, because a product of 1294 bp was obtained. It was cloned, sequenced and compared with the corresponding sequence of HSP70 gene (nt 3710–5341, the length of 1632 nt) of the GLRaV-1 isolate available in GenBank (AF195822) using the BLAST program (Altschul *et al*., 1990). The HSP70 sequence of the Czech isolate showed a high homology, confirming its identity. The obtained sequence is accessible in GenBank under Acc. No. AY644650.

*RT-PCR detection of GLRaV-1*

Results of the detection of GLRaV-1 by RT-PCR in 37 grapevine plants are shown in Table 1. The RT-PCR resulted in a product of expected length (540 bp). The specificity of the primers used was confirmed by sequencing of several products (data not shown).

As the virus was detected in 27 plants (11 of which were negative for the virus in the course of three years of yearly testing by DAS-ELISA), we consider the RT-PCR more sensitive than the DAS-ELISA for GLRaV-1 detection. In contrast, the RT-PCR did not detect the virus in the four plants, which were positive by DAS-ELISA (sv10, sv50, LPO70 and LPO79). The detection failure in those four cases could be caused by the sequence variability in the genome regions annealing the primers. To overcome this problem, we developed a molecular hybridization assay for GLRaV-1.

**Table 1. Detection of GLRaV-1, GLRaV-3, GFkV and GVA**

| Plant | Cultivar | GLRaV-1 | | | Other viruses detected by DAS-ELISA |
|---|---|---|---|---|---|
| | | Molecular hybridization probes | DAS-ELISA | RT-PCR | |
| sv 1 | Müller-Thurgau | + | + | ++ | |
| sv 2 | Müller-Thurgau | + | ++ | ++ | |
| sv 3 | Müller-Thurgau | (+) | – | ++ | GLRaV-3, GFkV |
| sv 4 | Müller-Thurgau | + | + | ++ | GVA |
| sv 5 | Müller-Thurgau | + | – | + | GLRaV-3, GFkV |
| sv 6 | Müller-Thurgau | + | +++ | ++ | GVA |
| sv 8 | Müller-Thurgau | ++ | – | – | |
| sv 9 | Müller-Thurgau | + | ++ | ++ | GLRaV-3, GFkV |
| sv 10 | Pinot Blanc | ++ | + | – | GFkV |
| sv 11 | Pinot Blanc | – | – | + | |
| sv 12 | Pinot Blanc | (+) | ++ | + | |
| sv 15 | Portugais Bleu | (+) | – | + | |
| sv 17 | Portugais Bleu | + | +++ | +++ | GVA |
| sv 20 | Pinot Blanc | ++ | ++ | ++ | GVA |
| sv 22 | Pinot Blanc | + | ++ | ++ | |
| sv 23 | Müller-Thurgau | - | – | – | GLRaV-3 |
| sv 26 | Müller-Thurgau | + | +++ | + | |
| sv 30 | Portugais Bleu | + | +++ | + | |
| sv 33 | Müller-Thurgau | + | – | + | GLRaV-3 |
| sv 34 | Müller-Thurgau | + | – | – | GLRaV-3 |
| sv 38 | Müller-Thurgau | ++ | – | + | GLRaV-3 |
| sv 39 | Müller-Thurgau | + | – | (+) | GLRaV-3 |
| sv 40 | Müller-Thurgau | + | – | + | GLRaV-3 |
| sv 41 | Müller-Thurgau | + | – | – | GLRaV-3 |
| sv 42 | Müller-Thurgau | + | – | + | GLRaV-3 |
| sv 50 | Gewürztraminer | + | + | – | |
| LPO 70 | Portugais Bleu | ++ | + | – | |
| LPO 72 | Saint Laurent | (+) | + | + | |
| LPO 73 | Müller-Thurgau | + | – | + | GLRaV-3 |
| LPO 74 | Müller-Thurgau | + | – | – | GLRaV-3 |
| LPO 77 | Müller-Thurgau | (+) | + | + | |
| LPO 78 | Silvaner | (+) | + | + | |
| LPO 79 | Silvaner | (+) | + | – | GLRaV-3 |
| LPO 82b | Pinot Blanc | (+) | + | + | |
| LPO 85 | Saint Laurent | + | + | + | GLRaV-3 |
| LPO 86 | Saint Laurent | + | – | + | |
| LPO 90 | Saint Laurent | (+) | – | – | GLRaV-3, GFkV |
| | Healthy grapevine | – | – | – | |

– = negative; (+) = probably positive (weak signal); + = positive (strong signal); ++ = highly positive (very strong signal); +++ = extremely positive (extremely strong signal).

*Molecular hybridization assay for GLRaV-1*

To improve further the detection of GLRaV-1 and to enable large-scale detection of the virus four non-radioactive RNA probes were prepared. Two (sense and antisense) 1294 nt long probes encompassed 79% of the HSP70 gene; two 540 nt long probes corresponded to the inner part of the HSP70 gene (Fig. 1), excluding a highly conserved phosphate 1 motif (Tian *et al*., 1996), located at the 5´-end of the gene of closteroviruses and eliminating non-specificity of longer probes. The results obtained with these probes are shown in Table 2.

The probes reacted with almost all 37 plants tested including five plants, which were negative for GLRaV-1 by ELISA and RT-PCR. All the four probes gave similar results; no differences were seen between short and long probes. However, the plants sv6, sv12 and sv26 were positive for GLRaV-1 only with both the antisense probes and the plant LPO 77 was positive with the long antisense probe only. These results indicated that the antisense probes were more sensitive than the sense ones. None of the probes reacted with a negative control (a virus-free grapevine). Comparison of the results obtained with the three detection methods is given in Table 1.

**Table 2. Detection of GLRaV-1 by molecular hybridization using various RNA probes**

| Plant | Cultivar | Probes | | | | Consensus |
|---|---|---|---|---|---|---|
| | | Antisense short | Sense short | Antisense long | Sense long | |
| sv 1 | Müller-Thurgau | + | + | + | + | + |
| sv 2 | Müller-Thurgau | + | (+) | + | (+) | + |
| sv 3 | Müller-Thurgau | (+) | (+) | (+) | – | (+) |
| sv 4 | Müller-Thurgau | + | + | ++ | (+) | + |
| sv 5 | Müller-Thurgau | + | + | + | + | + |
| sv 6 | Müller-Thurgau | (+) | – | ++ | – | + |
| sv 8 | Müller-Thurgau | ++ | ++ | ++ | ++ | ++ |
| sv 9 | Müller-Thurgau | + | + | (+) | (+) | + |
| sv 10 | Pinot Blanc | ++ | ++ | + | + | ++ |
| sv 11 | Pinot Blanc | – | – | – | – | – |
| sv 12 | Pinot Blanc | (+) | – | (+) | – | (+) |
| sv 15 | Portugais Bleu | (+) | (+) | (+) | (+) | (+) |
| sv 17 | Portugais Bleu | + | - | + | (+) | + |
| sv 20 | Pinot Blanc | ++ | ++ | ++ | + | ++ |
| sv 22 | Pinot Blanc | + | + | + | + | + |
| sv 23 | Müller-Thurgau | – | – | – | – | – |
| sv 26 | Müller-Thurgau | (+) | – | + | – | + |
| sv 30 | Portugais Bleu | + | (+) | + | + | + |
| sv 33 | Müller-Thurgau | + | + | + | (+) | + |
| sv 34 | Müller-Thurgau | + | + | + | + | + |
| sv 38 | Müller-Thurgau | ++ | ++ | ++ | ++ | ++ |
| sv 39 | Müller-Thurgau | + | + | + | (+) | + |
| sv 40 | Müller-Thurgau | + | + | + | + | + |
| sv 41 | Müller-Thurgau | + | + | + | + | + |
| sv 42 | Müller-Thurgau | + | (+) | ++ | (+) | + |
| sv 50 | Gewürztraminer | + | + | + | + | + |
| LPO 70 | Portugais Bleu | ++ | ++ | + | ++ | ++ |
| LPO 72 | Saint Laurent | (+) | (+) | (+) | (+) | (+) |
| LPO 73 | Müller-Thurgau | + | + | + | + | + |
| LPO 74 | Müller-Thurgau | + | + | (+) | + | + |
| LPO 77 | Müller-Thurgau | – | – | (+) | – | (+) |
| LPO 78 | Silvaner | (+) | (+) | (+) | – | (+) |
| LPO 79 | Silvaner | (+) | (+) | + | (+) | (+) |
| LPO 82b | Pinot Blanc | (+) | (+) | (+) | – | (+) |
| LPO 85 | Saint Laurent | + | + | + | + | + |
| LPO 86 | Saint Laurent | + | + | + | + | + |
| LPO 90 | Saint Laurent | (+) | (+) | + | (+) | (+) |
| | Healthy (control) | – | – | – | – | – |

– = negative (no signal); (+) = probably positive (weak signal); + = positive (strong signal); ++ = highly positive (very strong signal).

In conclusion, in 36 out of 37 grapevine plants with leafroll symptoms tested the presence of GLRaV-1 was detected by at least one method, mostly the molecular hybridization. The only plants negative for GLRaV-1 was positive for GLRaV-3. The mixed infection of GLRaV-1 and GLRaV-3 occurred in11 plants, that of GLRaV-1 and GVA in 4 plants, and that of GLRaV-1 and GFkV in 1 plant. A multiple mixed infection of GLRaV-1, GLRaV-3 and GFkV occurred in 4 plants.

## Discussion

Detection of GLRaV-1 is a basic step in establishing a healthy prebasic propagation material of grapevine cultivars. However, the virus is often present in infected grapevines at a very low concentration, and, like other grapevine closteroviruses, it is very difficult to purify (Gugerli *et al*., 1984). Antisera prepared against this virus are usually of a low titer and need to be absorbed with a healthy plant extract before use in ELISA (Hu *et al*., 1990).

For this reason, we developed more sensitive methods based on detection of viral RNA and compared them with detection by DAS-ELISA. We obtained contradictory results in the case of five grapevines plants (sv8, sv34, sv41, LPO74, and LPO90), which were positive for GLRaV-1 by one of the three methods only – the molecular hybridization. These results should be considered insignificant, because four of the five "problematic" plants were found GLRaV-3-positive. However, the plant sv23, which was positive for GLRaV-3, was negative for GLRaV-1 by all the three detection methods for GLRaV-1. We can conclude that the probes used in the molecular hybridization reacted correctly: positively with GLRaV-1 and negatively with GLRaV-3, which is phylogenetically close to GLRaV-1. Moreover, the plant sv8 was negative for all the viruses tested but positive by molecular hybridization using all the four probes.

There are two possible explanations. (i) The probes reacted with another virus, which was not covered by the assay, e.g. GLRaV-4 or GLRaV-8 (antisera for those viruses are not commercially available). However, the sequence analysis of GLRaV-4 reveals large differences in the HSP70 gene sequence between these two viruses and a non-specific annealing of the probes to target sequences under stringent washing conditions of hybridization is improbable. The sequence of GLRaV-8 HSP70 gene is not available. The existence of other leafroll viruses should be considered, however, the possibility that they share a high nucleotide sequence similarity enabling to react with probes under stringent conditions is highly improbable.

(ii) The probes reacted correctly with GLRaV-1, but the sensitivity of the assay was higher than that of RT-PCR, probably due to some degree of variability in the HSP70 gene, especially in the primers' regions. This phenomenon can make the detection by RT-PCR with the primers used less reliable. A high degree of variability has been described by Little *et al*. (2001) for GLRaV-1 HSP70, CPd1 and CPd2 genes with mutations in up to 60% of nucleotide positions.

Therefore, the higher sensitivity of molecular hybridization compared to RT-PCR seems to explain the observed contradictory results. Molecular hybridization with digoxigenin-labeled RNA probes has been reported by Ivars *et al*. (2004) to detect 0.03 pg of a viral transcript and 0.1 pg of a purified virus (Pelargonium flower break virus and Pelargonium line pattern virus), indicating a 125 times higher sensitivity than ELISA. A high sensitivity has been reported for digoxigenin-labeled RNA probes by Más *et al*. (1993) with a detection limit of 250 pg of purified RNA of Cherry leaf roll virus.

During our study, we observed some differences in the sensitivity of differently oriented RNA probes. It is possible that (i) the antisense probes reacted with viral ssRNA as well as the plus strand of dsRNA, present in the RNA isolated from infected grapevine plants and (ii) the sense probes reacted with the minus strand of dsRNA only and had less targets for hybridization compared to the antisense probes. But the sensitivity of the detection method could minimize the differences between the results obtained with the sense and antisense probes.

We conclude that a highly sensitive molecular hybridization method for the detection of GLRaV-1 was developed using digoxigenin-labeled RNA probes. The method seems to be more reliable for the detection of the virus than DAS-ELISA or RT-PCR, as it can overcome high variability in the genome region annealing the primers, which can make the RT-PCR detection unreliable. In the absence of detailed information on GLRaV-1 genome variability in the central European area, a simultaneous application of several methods is still desirable for specific and reliable detection of this severe pathogen.

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## References

Altschul SF, Gish W, MillerW, Myers EW, Lipman DJ (1990): Basic local alignment search tool. *J. Mol. Biol*. **215**,403–410.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2003): GenBank. *Nucleic Acids Res.* **31**, 23–27.

Boscia D, Greif D, Gugerli P, Martelli GP, Walter B, Gonsalves D (1995): Nomenclature of grapevine leafroll-associated putative closteroviruses. *Vitis* **34**, 171–175.

Dovas CI, Katis NI (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. *J. Virol. Methods* **109**, 217–226.

Fazeli CF, Rezaian MA (2000): Nucleotide sequence and organization of ten open reading frames in the genome of Grapevine leafroll-associated virus 1 and identification of three subgenomic RNAs. *J. Gen. Virol.* **81**, 605–615.

Gugerli P, Brugger JJ, Bovey R (1984): L'enroulement de la vigne: mise en évidence de particules virales et développement d'une méthode immunoenzymatiques pour le diagnostic rapide. *Rev. Suisse Vitic. Arboric. Hortic.* **16**, 299–304.

Habili N, Ewart AJW, Fazeli CF, Scott NS, Krake LR, Rezaian MA (1996): Virus types associated with grapevine leafroll disease in Australia. *Aust. Grapegrower and Winemaker* **390a**, 25–28.

Hu JS, Gonsalves D, Boscia D, Namba S (1990): Use of monoclonal antibodies to characterize grapevine leafroll associated closteroviruses. *Phytopathology* **80**, 920–925.

Ivars P, Alonso M, Borja M, Hernández C (2004): Development of a non-radioactive dot-blot hybridisation assay for the

detection of Pelargonium flower break virus and Pelargonium line pattern virus. *Eur. J. Plant Pathol.* **110**, 275–283.

Komínek P, Holleinová V (2003): Evaluation of sanitary status of grapevines in the Czech Republic. *Plant Soil Environ.* **49,** 63–66.

Little A, Fazeli CF, Rezaian MA (2001): Hypervariable genes in Grapevine leafroll associated virus 1. *Virus Res.* **80**, 109–116.

Martelli GP, Agranovsky AA, Bar-Joseph M, Boscia D, Candresse T, Coutts RHA, Dolja VV, Falk BW, Gonsalves D, Jelkmann W, Karasev AV, Minafra A, Namba S, Vetten HJ, Wisler GC, Yoshikawa N (2002): The family Closteroviridae revised. *Arch. Virol.* **147**, 2039–2044.

Más P, Sanchez-Navarro JA, Sanchez-Pina MA, Pallás V (1993): Chemiluminescent and colorigenic detection of cherry leaf roll virus with digoxigenin-labeled RNA probes. *J. Virol. Methods* **45**, 93–102.

Rowhani A, Walker MA, Rokni S (1992): Sampling strategies for the detection of grapevine fanleaf virus and the grapevine strain of tomato ringspot virus. *Vitis* **31**, 35–44.

Rowhani A, Uyemoto JK, Golino DA (1997): A comparison between serological and biological assays in detecting grapevine leafroll associated viruses. *Plant Dis.* **81**, 799–801.

Rozen S, Skaletsky HJ (2000): Primer3 on the WWW for general users and for biologist programmers. In Krawetz S, Misener S (Eds): *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp. 365–386.

Seddas A, Haidar MM, 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 Pathol.* **49**, 80–85.

Sforza R, Boudon-Padieu E, Greif C (2003): New mealybug species vectoring Grapevine leafroll-associated viruses-1 and -3 (GLRaV-1 and -3). *Eur. J. Plant Pathol.* **109**, 975–981.

Tian T, Klaassen VA, Soong J, Wisler G, Duffus JE, Falk BW (1996): Generation of cDNA specific to Lettuce infectious yellows closterovirus and other whitefly-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.

Woodham RC, Antcliff AJ, Krake LR, Taylor RH (1984): Yield differences between Sultana clones related to virus status and genetic factors. *Vitis* **23**, 73–83.

Zimmermann D, Bass P, Legin R, Walter B (1990): Characterization and serological detection of four closterovirus-like particles associated with leafroll disease of grapevine. *J. Phytopathol.* **130**, 205–218.