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ORIGINAL ARTICLE

Improved detection, mealybug transmission and prevalence of grapevine Roditis leaf discoloration-associated virus in Greek vineyards

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Abstract

Grapevine Roditis leaf discoloration-associated virus (GRLDaV) is a member of the species *Badnavirus decoloratiovitis* in the genus *Badnavirus* that was recently identified in grapevine using high throughput sequencing. In this study, a large-scale survey was conducted to monitor the prevalence of the virus in major viticultural areas of Greece. A total of 970 samples were collected from vines and molecularly analysed using PCR assays targeting two regions of the virus genome. GRLDaV was detected in 8 geographic areas of Greece, showing high frequency in southern and central Greece and an overall prevalence of 17.1%. GRLDaV was identified mainly in autochthonous grapevine varieties, and it showed higher frequency in the self-rooted ones, especially in the geographic region of the Cyclades islands. In transmission tests conducted using *Planococcus ficus* (Signoret) and 48 h acquisition access period (AAP) and inoculation access period (IAP), the virus was transmitted with an efficiency of 14.3% (3/21 grapevines). Finally, electron microscopy revealed the bacilliform morphology of GRLDaV virions, thus further confirming its episomal infection.

Keywords Badnavirus · Grapevine · GRLDaV distribution · Mealybug transmission · electron microscopy

Introduction

The genus *Badnavirus* (family *Caulimoviridae*) includes viruses that have non-enveloped bacilliform virions with a monopartite 7.2 to 9.2 kb long dsDNA genome containing three to seven open reading frames (ORFs) (Bhat et al. 2016). These so-called pararetroviruses can infect and cause considerable losses in many crops, including grapevine, in which three badnavirus species were recently reported. Grapevine vein clearing virus (GVCV) was the first badnavirus identified in grapevine in the USA in 2011 (Zhang et al. 2011), followed by grapevine Roditis leaf discoloration-associated virus (GRLDaV) (Maliogka et al. 2015) and grapevine badnavirus 1 (GBV-1) (Vončina and Almeida 2018).

GRLDaV was identified in 2015 in a twenty-year-old Roditis vine showing typical leaf discoloration symptoms, using high throughput sequencing (HTS) of small interfering RNAs (Maliogka et al. 2015). This study unveiled the putative association of GRLDaV with Roditis leaf discoloration (RLD), a graft-transmissible grapevine disease first reported in Greece in the 1980s (Rumbos and Avgelis 1989) and considered to have an unknown etiology until 2015. Since its first identification, GRLDaV has been reported in Italy (Chiumenti et al. 2015, 2016), Turkey (Ulubas Serçe et al. 2018), Croatia (Vončina and Almeida 2018) and South Africa (Bester et al. 2021), where it was identified primarily on autochthonous grapevine varieties using different HTS approaches, except for the South African isolate which was identified in a German cultivar maintained in a germplasm collection. Based on the currently available complete sequences, the circular dsDNA genome of GRLDaV ranges between 6988 and 7188 nucleotides (nts) in length and includes four ORFs (ORF1-4). ORF1, ORF2 and ORF4 encode proteins with presently unknown functions, whereas ORF3 encodes a large polyprotein containing

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motifs related to virus replication, encapsidation, and movement (Maliogka et al. 2015).

Given its recent identification in several grapevine-growing countries, the European and Mediterranean Plant Protection Organization (EPPO) included the virus in the pest Alert list in October 2018. In Greece, where GRLDaV was initially identified, it was associated, as mentioned above, with Roditis leaf discoloration disease (Maliogka et al. 2015; Rumbos and Avgelis 1989). On the contrary, in all the other countries where it was reported, the virus was identified in vines having various symptoms (Bester et al. 2021 Vončina and Almeida 2018; Ulubas Serçe et al. 2018; Chiumenti et al. 2016). So far, data on the biology, geographic distribution, prevalence in the vineyards, and transmission manner of the virus are rather limited. Although it is known that several badnaviruses infecting different plant hosts are transmitted by mealybugs (Tsai et al. 2010; Meyer et al. 2008; Macanawai et al. 2005; Bhat et al. 2003), and it has been reported that *Planococcus citri* and *Pseudococcus viburni* are able to acquire GLRDaV (Moran et al. 2020), as it was detected molecularly in their body, there is no proof whether these or other mealybug species can transmit the virus.

Therefore, this study aimed to shed light on the biological and epidemiological traits of GRLDaV by carrying out a large-scale survey in major viticultural areas of Greece. In addition, its transmission with the mealybug species *Planococcus ficus* (Signoret), commonly found in Greek vineyards, was studied. The application of electron microscopy on infected grapevine extracts revealed the bacilliform morphology of the virions of GRLDaV, thus further confirming its episomal infection.

Materials and methods

Plant material and nucleic acids extraction

In 2016–2022, a total of 970 grapevine samples randomly collected from 48 different vineyards in Greece were analysed (Table 1). In some cases, virus-like symptoms were observed even though not systematically recorded. Leaves and/or canes from grafted foreign varieties (202 samples from 20 varieties), self-rooted (207 samples from 50 varieties) and grafted (507 samples from 30 varieties) autochthonous varieties and rootstocks (34 samples from 19 different rootstocks) were collected during different seasons (Table 1). The surveys took place mainly in commercial vineyards and secondly in the grapevine germplasm collections of the Aristotle University of Thessaloniki (AUTH), the Agricultural University of Athens (AUA), and the National Grapevine Institute (NGI) in Athens (Lykovrisi, Attica). A collection of samples of autochthonous self-rooted grapevine varieties from the Cyclades islands maintained in the Laboratory of Plant Pathology (School of Agriculture, Aristotle University of Thessaloniki) was also tested.

Total nucleic acid (TNA) was extracted from either cane phloem tissue scrapings or leaves with petioles, depending on the sampling period. More particularly, cane phloem tissue and scrapings were used during fall and winter, whereas leaves with petioles were used during spring and the beginning of summer. The extraction was performed using a CTAB based method according to a modified protocol of Doyle and Doyle (1987). In short, the grapevine tissue was ground in a 1:10 v/w ratio with the CTAB buffer. For the final step, the pellets were washed with 250 μl of 70% ethanol each time, and finally, they were resuspended in 50 μl of RNase free water.

Molecular detection of GRLDaV

Initially, the detection of GRLDaV was based on the previously designed primer pair targeting ORF 3 (Maliogka et al. 2015). As soon as new virus sequences became available, *in silico* analysis indicated that those primers could not detect all virus isolates. To further improve the detection method, new primers were designed in the RT region (Table 2), which is considered conserved for the genus *Badnavirus*, using an alignment of the already deposited complete genome sequences in GenBank (HG940503.2, KT965859.1, MT783680.1, MF991952.1). The homologous sequences of the genetically related grapevine badnavirus 1 (GBV-1) (MF781082.1) and fig badnavirus 1 (FBV-1) (JF411989.1, MK348055.1, MW522617.1) were included in the alignment. Another pair of primers were also designed at

Table 1 Incidence of GRLDaV in self-rooted, grafted autochthonous varieties, foreign varieties, and rootstocks

Sample type	Total samples	GRLDaV Positive	GRLDaV positive percentage
Self-rooted autochthonous (S)	207	67	32.4%
Grafted autochthonous (G)	526	96	18.5%
Foreign grafted (F)	202	2	0.9%
Rootstocks (R)	35	2	5.7%
SUM	970	160	17.1%

Table 2 Primers designed for the detection of grapevine Roditis leaf discoloration-associated virus

Primer name	Primer Sequence (5'-3')	Amplified Region	Ta*	Amplicon's length (bp)
Rt-F	AGTCGTCATCGAACAACTGCAATGC	RT	60	271
Rt-R	GGTACCCAGAATGCTGTCCAYTC			
BadcircleF	AAGAATACTACAACTCAGAGAT	t- RNA binding	55	197
BadcircleR	AGT TTG AAG AAA TAA ATT AGA GGT			

*Ta: annealing temperature

the region flanking the tRNA binding site (Table 2), where a conserved area was also identified according to the alignment of the complete genome sequences. A comparison of the detection range of the primer pair targeting the ORF3 region by Maliogka et al. (2015) with those targeting the tRNA (named Bad circle primers) and RT regions designed herein was performed using isolates previously characterized through HTS (Suppl. Figure 1). Isolates of FBV-1 and GBV-1 were also tested using the herein developed primers.

PCRs were performed in a 20 µl reaction volume using 2 µl of DNA (30–50 ng/ul), 0.4 µM of each primer, 10 mM Tris HCl (pH 9), 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1.5 U GRS Hot Start Taq DNA polymerase (GRiSP Research Solutions) and DEPC-treated water. The cycling conditions were 94 °C for 5 min as a preliminary denaturation step, followed by 40 cycles at 94 °C for 30 s, Ta°C of each pair of primers (Table 2) for 30 s, 72 °C for 30 s, and the final extension step at 72 °C for 2 min according to the product expected. PCR products were analysed with electrophoresis using a 1.5% agarose gel previously stained with Midori Green Advance gel (Nippon, Dueren, Germany) and visualized under UV light.

Selected amplicons of the PCRs obtained from 5 GRLDaV isolates previously characterized through HTS were Sanger sequenced by purifying the DNA using the NucleoTrap® purification kit (Macherey-Nagel Duren, Germany) according to the manufacturer's instructions and sequencing at Eurofins Genomics (Wien, Austria) or Macrogen Europe (The Netherlands) in both directions. The sequences were analysed with Geneious prime (version 2022 1.1) to confirm primers' specificity.

Mealybug transmission assays

Typing of mealybug species and colony maintenance

Mealybugs of the species *Planococcus ficus* (Signoret) were collected in July 2020 from a vineyard in Kavala, in northern Greece, and kept in potato sprouts under lab conditions (24–26 °C, 12 h dark). A brief DNA extraction protocol proposed by Pusz-Bochenska et al. (2020), followed by PCR (Malausa et al. 2011), and amplicon sequencing was conducted for mealybug identification. The molecular identification tests were initially carried out three months after the collection and colony establishment and then periodically each month for four more months to confirm the purity of the colony.

The extraction was performed as follows. Approximately three instars of the 2nd and 3rd developmental stage were smashed between two Whatman® FTA® cards; the cards were then cut into round shapes with the mealybugs in them and placed in a 1.5 ml sterilised tube. 100 µl of Lysis buffer (Chatzinasiou et al. 2010) was placed in the tube, and the FTA cards were smashed again with a tip. The FTA cards were transferred with a sterilized tweezer into another 1.5 ml RNase DNase free tube, where a small hole was already opened at the bottom to make it a homemade column. The cards were washed twice with 100 µl Wash II Buffer (Chatzinasiou et al. 2010) and were centrifuged at 10.000 xg for 30 s. The homemade column was left to dry for 3 min, and DNA was eluted with 40 µl of DEPC-treated water.

Transmission trials of GRLDaV with *P. ficus*

A 30-year-old vine cv. Platani (code Rod 2), infected with GRLDaV as well as with grapevine leafroll-associated virus 3 (GLRaV-3), was used as a virus source for the transmission tests. Twenty-one-rooted vine cuttings of pre-basic material (as defined by commission implementing directive 2014/98/Eu) of the Greek white variety Vidiano were established in pots in March 2021 in open environmental conditions (terrace) and were used as recipient plants for virus transmission. The cv. Vidiano vines, prior to the transmission trials, were tested by PCR or RT-PCR for the presence of GRLDaV using the RT primers developed herein and for GRLaV-3, as suggested by Bester et al. (2014). To determine the sufficient AAP time for the *P. ficus* – GRLDaV combination, in May 2021, 2nd and 3rd stage instars were given either 24 or 48 h AAP on a GRLDaV-infected grapevine detached leaf of cv. Platani. After that, to detect the virus, TNA extraction was performed on a pool of 3 mealybugs used in each AAP, followed by PCR using the RT targeting primers. GRLDaV was only detected in the pool of mealybugs given a 48 h AAP.

Therefore, in all subsequent transmission tests, 2nd and 3rd stage instars of *P. ficus* were given a 48 h AAP with 15 instars placed on each infected source leaf. Subsequently,

one infected leaf carrying the 15 mealybugs was transferred onto a leaf of each of the twenty-one healthy cv. Vidiano vines where it remained for an IAP of 48 h, and then the vines were sprayed with imidacloprid-based insecticide. Every month after the transmission at the end of May 2021 and for a period of four months, the cv. Vidiano recipient vines were tested for the presence of GRLDaV with PCR assays using the primers targeting the RT, ORF3 and tRNA regions. PCR amplicons were further sequenced as described above to confirm the presence of GRLDaV at the recipient plants. Molecular detection was repeated monthly from May to October 2022 and one last time in February of 2023.

Transmission electron microscopy (TEM)

Virus particles were purified from mature leaves as described previously (Gugerli 1984). Twenty g of GRLDaV infected grapevine leaves cv. Roditis were homogenized, filtered and enriched by ultracentrifugation. The enriched fractions were negatively stained using 2% uranyl acetate, and virions were observed using a Tecnai Spirit BioTWIN (FEI).

Results

Molecular detection of GRLDaV

The herein-developed assays using primers targeting either the RT or the tRNA binding domains exhibited a higher detection range compared to the primers targeting ORF3 previously developed in our lab (Maliogka et al. 2015) (Fig. 1). *In silico* analysis indicated that the new primers do not anneal to the sequence of the genetically related FBV-1 and GBV-1 and this was further shown *in vitro*. Sanger sequencing of the amplicons obtained from GRLDaV-infected vines confirmed the specificity of the new primers. More specifically, the sequences obtained from the RT region of the five GRLDaV isolates showed 84.9–95.6% nucleotide (nt) identity to the virus isolates deposited in GenBank and 81.3- 83.6% to the FBV-1 isolates (accession Nos. JF411989.1, MK348055.1, MW522617.1) and 72-72.4% to the GBV-1 isolate (MF781082.1). Likewise, the GRLDaV sequences from the tRNA region displayed 80–96% nt identity to homologous GRLDaV sequences from GenBank and only 57.8%-59.4% nt identity to the FBV-1 isolates and 48.3%-52.4% to the GBV-1 Croatian isolate (Supl. Table 1a, b). Therefore, the novel primers were subsequently adopted and used for the reliable detection of the virus in the grapevine material.

Prevalence of GRLDaV in different geographic regions and grapevine types

GRLDaV was detected in 8 geographic regions of Greece (Fig. 2, Suppl. Table 2), with an overall incidence of 17.1% (160/970 samples). The virus showed high frequency in southern (Peloponnese) and central Greece (Larisa, Voiotia, Trikala, Magnesia, Attica) (Suppl. Table 2), as it was identified in 16.4% (21/128) and 25.6% (63/246) of the tested vines, respectively. In Attica, the percentage of GRLDaV positive accessions in the grapevine germplasm collection of NGI was 37.7% (63/167). In Crete and the Cyclades islands,

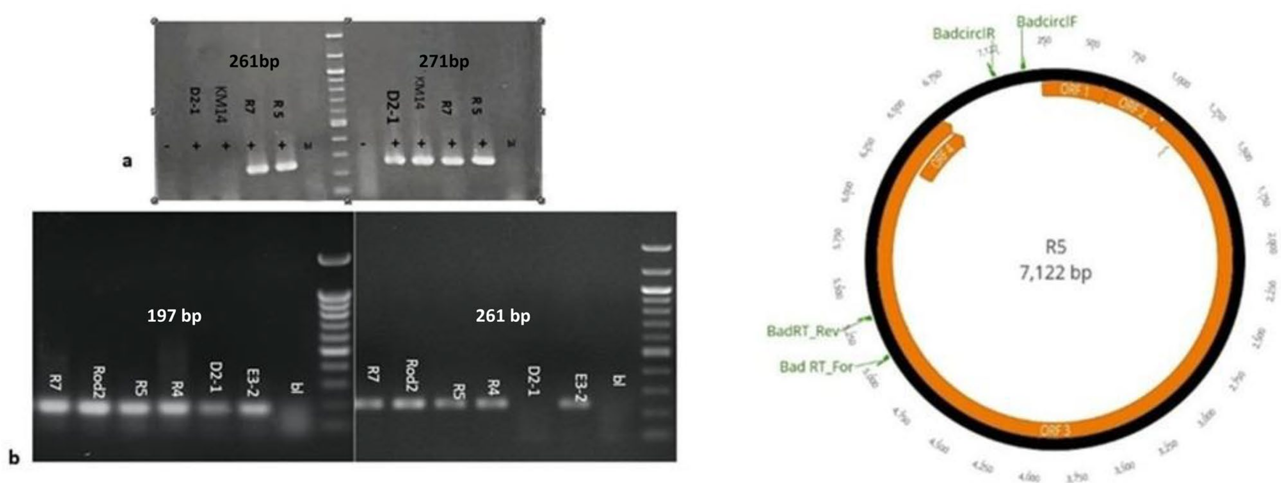

Fig. 1 **a**, **b** Agarose gel electrophoretic analysis of PCR products obtained using **a** primers targeting the ORF3 of GRLDaV (Maliogka et al. 2015) (left panel) and RT primers designed in this study in 4 different GRLDaV isolates and **b** primers (Badcircle primers) targeting the T-RNA binding region designed in this study (left panel) and ORF3 targeting primers (Maliogka et al. 2015) (right panel) in 6 different GRLDaV isolates. **c** Genome organization of GRLDaV indicating the annealing sites of the primer pairs used for virus detection

GRLDaV was identified in 16.6% (7/42) and 39.4% (65/165) of the tested vines, whereas in the other surveyed areas, the virus presence was very low.

The virus was detected in higher frequency in samples of the self-rooted autochthonous varieties (32.4%, 67/207 samples), compared to the grafted autochthonous, where it was 18.5% (96/526). In the foreign grafted varieties and rootstocks, the incidence of GRLDaV was 0.9% (2/202) and 5.7% (2/35), respectively (Table 1). More specifically, GRLDaV was identified in 26/50 different self-rooted autochthonous and 20/30 grafted autochthonous varieties, as well as in 2/20 foreign varieties and 2/19 different rootstocks tested.

Among the autochthonous varieties, the highest GRLDaV prevalence was recorded in the samples from Cyclades (39.4%, 65/165), which originated from 20 of the 30 different self-rooted varieties tested from this area. The grafted autochthonous varieties in Korinthos (Southern Greece Peloponnese) showed a virus presence of 32.7% (17/52) (3/11 varieties tested). In Ilia, 4 out of 24 samples were positive (2 different grafted autochthonous varieties out of the 6 tested). Both foreign grafted varieties in which GRLDaV was detected originated from northern Greece, one from Thessaloniki (vineyard TH2, cv. Chardonnay) and the other one from Florina (F2) (Suppl. Table 2). Finally, GRLDaV was detected in two (8B and 779 Paulsen) of the eight rootstocks tested from the grapevine germplasm collection of the School of Agriculture in Thessaloniki (vineyard Th1*).

Mealybug transmission

Initial PCR testing showed that *P. ficus* could acquire GRLDaV after a 48 h AAP on the GRLDaV-infected plants. Therefore, the 15 mealybugs placed onto each leaf (Fig. 3) were given 48 h AAP and IAP in all subsequent experiments. GLRaV-3 was utilised as a control for testing the vectoring ability of *P. ficus*, and was detected in early October of 2021 in four out of the 21 cv.

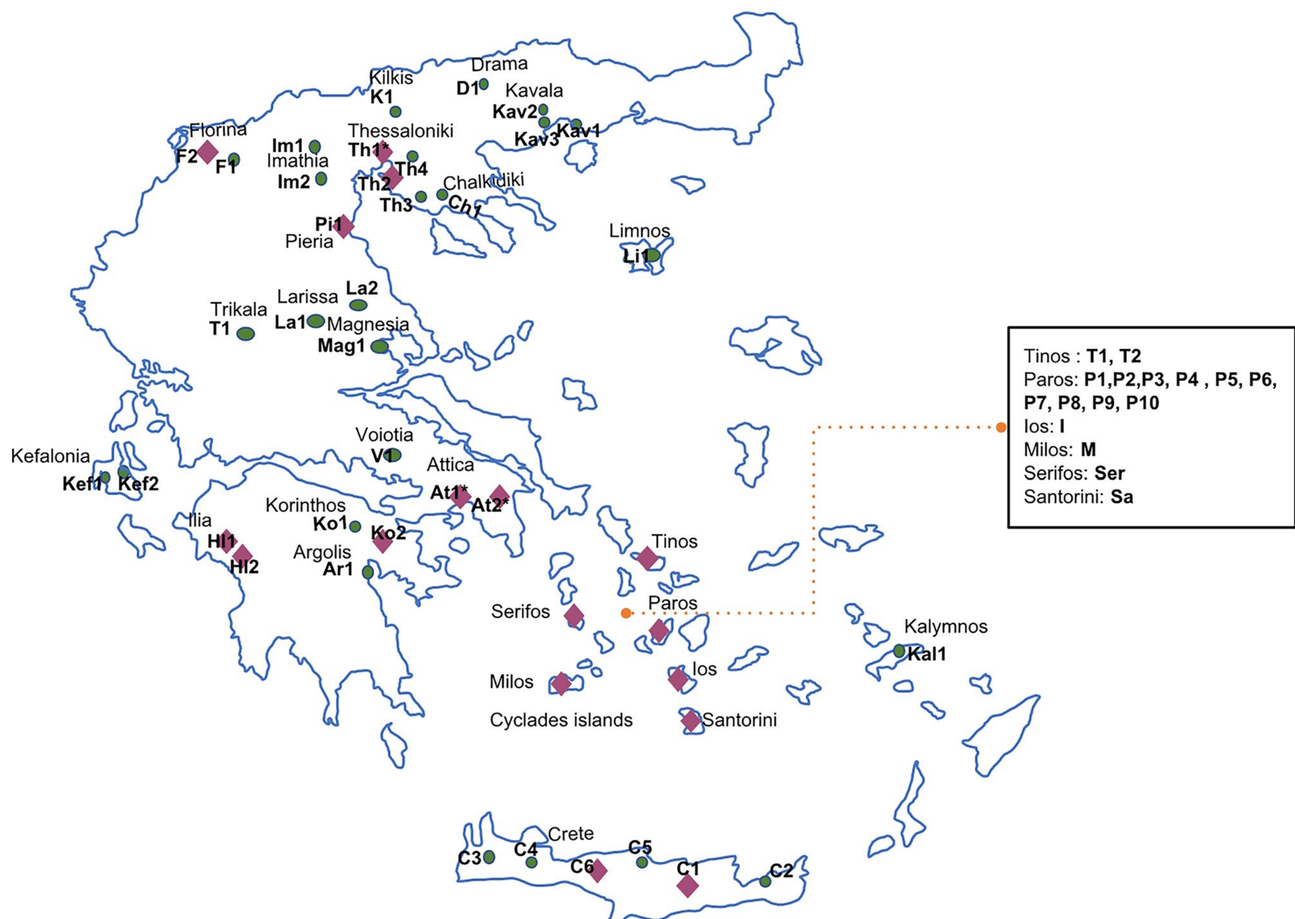

Fig. 2 Map of Greece showing the surveyed areas. The regions with the green circle (●) are the surveyed vineyards where the virus was not identified, and the regions with red rhombus (◆) are the vineyards where grapevine Roditis leaf discoloration associated virus was identified

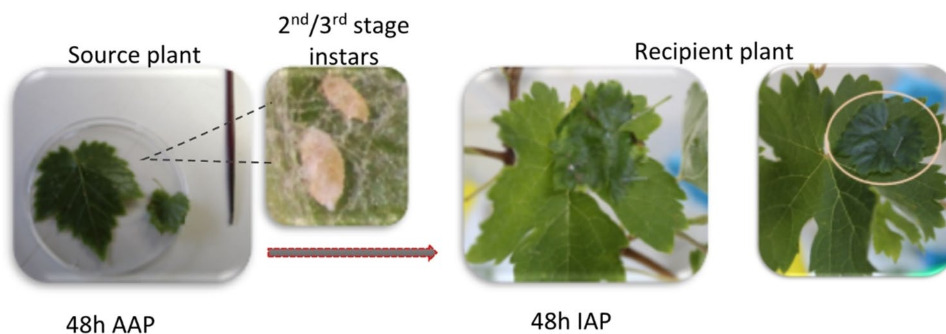

Fig. 3 Workflow of the transmission trials of grapevine Roditis leaf discoloration associated virus using instars of *Planococcus ficus*. The instars were placed in the source plant for 48 h AAP and in the recipient plant for 48 h IAP

Vidiano plants. At the same time, two of these recipient plants were found to be infected with GRLDaV. The presence of GRLDaV on the infected Vidiano vines was verified using the two different PCR assays developed herein targeting different regions of the virus genome. In May 2022, one more plant tested positive for GRLDaV. The three infected vines were continuously monitored and found GRLDaV positive until February 2023. Further Sanger sequencing of the amplicons revealed 100% nt identity with the respective genomic regions of the GRLDaV isolate in the source plant (Rod2). Interestingly, leaf discoloration symptoms were observed one year after inoculation (May 2022) in all three plants, possibly attributed to GRLDaV. In addition, leafroll symptoms, possibly due to the presence of GLRaV-3 (Fig. 4), appeared in the fall of the same year in the four infected vines. Further HTS analysis of the infected vines could clarify whether other viruses are also present in the vines affecting the observed symptomatology.

Transmission electron microscopy

Few bacilliform particles with an estimated average length of 110 nm were observed by TEM, and the coinfection status of accession R5 (GLRaV-3 and GLRDaV) was confirmed (Fig. 5).

Discussion

GRLDaV is one of the three known badnaviruses infecting grapevine worldwide. Due to its recent reports from several countries, it has been included in the EPPO alert list; however, the data on its transmission mechanism(s), epidemiology, and spread are limited. In this study, novel PCR assays were developed using primers targeting conserved regions of the currently available complete genome sequences of GRLDaV isolates originating from Greece, Italy, and South Africa. These assays showed a high detection efficiency and, therefore, were

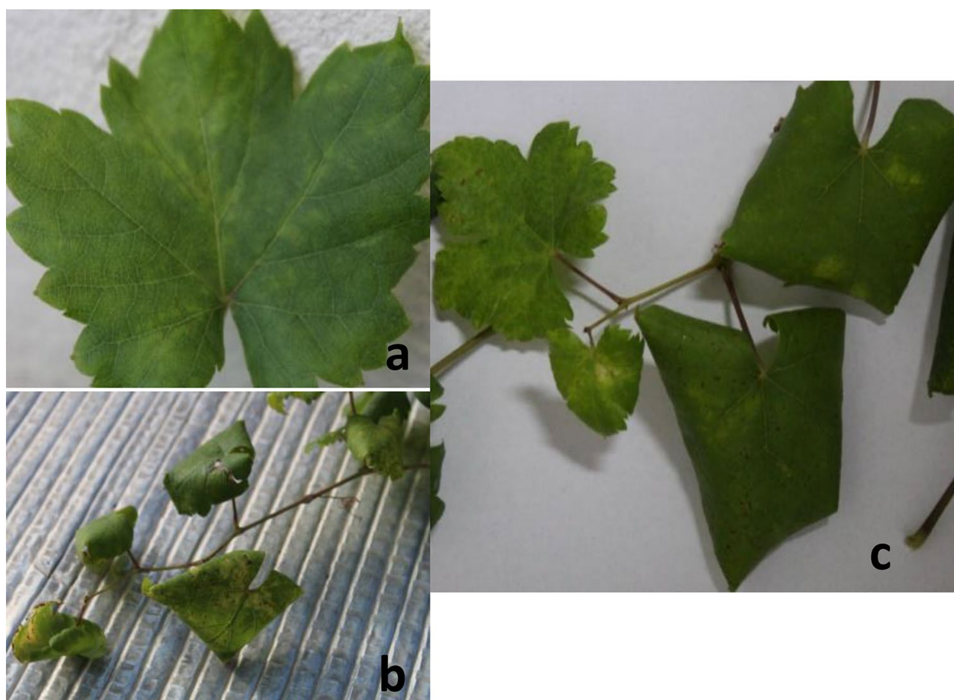

Fig. 4 Leaf discoloration (**a**) from May of 2022 and leafroll symptoms (**b**, **c**) from late fall of 2022 on leaves of Vidiano grapevine variety one year after transmission trials

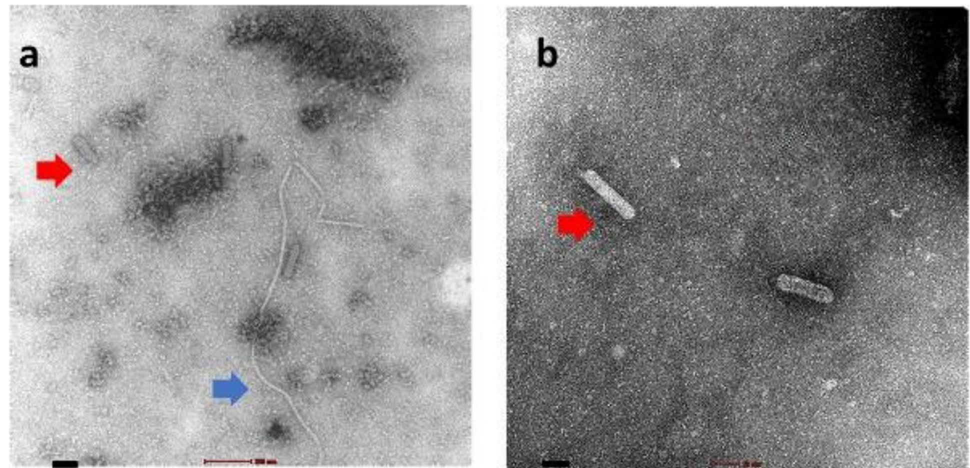

Fig. 5 Bacilliform virions of grapevine Roditis leaf discoloration associated virus (red arrow in **a** and **b**). Filamentous virion (GLRaV-3) coinfecting the tested plant (blue arrow in **a**). A black segment indicates the scale (**a**: 100 nm, **b**: 50 nm)

used for monitoring the virus prevalence in Greek vineyards. This is the first large-scale survey conducted in a country to monitor the distribution of GRLDaV-infected plants.

GRLDaV was identified in several regions of Greece with an exceptionally high prevalence at the Cyclades islands and in the germplasm collections of NGI and AUA in Attica, including plant material of diverse geographic origins. Interestingly, virus presence was higher in the autochthonous Greek varieties, which comes in agreement with the reports concerning its presence in local varieties from other countries and mainly from the Mediterranean basin (Chiumenti et al. 2015; Ulubas Serçe et al. 2018; Voncina and Almeida 2018; Bester et al. 2021). Similarly, recent studies on the spread of GBV-1, another grapevine-infecting badnavirus, in Croatia and the USA indicated its wide distribution in autochthonous cultivars in the coastal wine-growing region of the country (Jagunić et al. 2022a).

The detection of GRLDaV in several geographic regions, with a relatively high prevalence in some of them, indicates its putative presence in Greek vineyards for a long time. In fact, the exceptionally high rates of GRLDaV in self-rooted local grapevine varieties at the Cyclades islands underline a putative implication of a vector in the vine-to-vine transmission of the virus. It is well known that most badnaviruses are transmitted by mealybugs and rather few by aphids in a semi-persistent manner (Bhat et al. 2016). Recent studies in Croatia showed that GBV-1 is transmitted efficiently (61%) under laboratory conditions between grapevines by the mealybug species *P. ficus* (Jagunic et al. 2022b). Concerning GRLDaV, Moran et al. (2020) showed virus acquisition by *P. citri* and *Pseudococcus viburni* based on the direct application of real-time PCR on the mealybugs fed on the infected grapevine without any further tests on plants to verify its transmission.

Our study investigated whether *P. ficus* (Signoret) populations, known to infest the Greek vineyards since 1956 (Ezzat and McConnell 1956), can transmit the virus. The results showed successful transmission of GRLDaV to the white grapevine cv. Vidiano by the *P. ficus* mealybug species with an efficiency of 14.3%. This significant finding advances our current knowledge of the factors affecting GRLDaV's epidemiology. Interestingly, the high presence of GRLDaV in the vineyards of NGI, AUA, and the vineyards of Korinthos (Milonas et al. 2008) is putatively associated with the presence of mealybugs. In contrast, the absence of mealybugs in the vineyard of AUTH in Thessaloniki, where the virus presence was lower (Suppl. Table 2), strongly supports the implication of mealybugs in the spread of GRLDaV.

The first evidence of grapevines and fig trees cultivated in the same zone dates to the Late Bronze Age (Valamoti et al. 2020). Recently, Chirkov et al. (2022) reported that several divergent GBV-1 isolates (fGBV-1) were found in different species of fig trees exhibiting 84.6% nt identity with GBV-1 at the conserved RT/RNaseH region, showing possible epidemiological interaction between these two hosts. Moreover, the fact that the mealybug *P. ficus* (Signoret) can feed on fig trees (Ezzat and McConnell 1956; Granara et al. 1997) and the existence of FBV-1, which is phylogenetically close to GRLDaV (Maliogka et al. 2015), in fig, indicate a possible transmission of a GRLDaV ancestor from grapevine to fig trees or vice-versa, by mealybugs.

Finally, bacilliform virions of GRLDaV, typical of the genus *Badnavirus*, were observed by TEM for the first time, thus confirming its episomal infection and the size of the virions length of 110 nm. Overall, our study provides new data on the prevalence and epidemiology of GRLDaV that could be used to design suitable strategies for virus control. Future studies should try to unveil whether other mealybug species are also implicated in the spread of GRLDaV under field conditions.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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