Hungarian University of Agriculture and Life Sciences

Thesis of doctoral (PhD) dissertation

Virological investigation of grapevine plantations using small RNA-based high-throughput sequencing and identification of new hosts of Grapevine Pinot gris virus in Hungary

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1. BACKGROUND AND OBJECTIVES

Grapevine cultivation has an important economic and cultural role worldwide. Europe, including Hungary, is a key region for grape and wine production. Grapevines are cultivated not only for fresh consumption but also for several processed products, among which wine is the most important. The success of grape production is influenced by various biotic and abiotic stress factors, of which viral and viroid infections can cause significant economic losses.

Control of viruses and viroids infecting grapevine is limited, as infected plants cannot be cured and the pathogens can persist for long periods in propagation material and vineyards. Viral infections may lead to reduced yield, deterioration of fruit quality, and gradual decline of vineyards. Therefore, prevention, the use of pathogen-free propagation material, and the early and reliable detection of infections form the basis of virus control in grapevine production.

In Hungary, several diagnostic methods are available for the detection of viral and viroid infections of grapevine, including biological indexing, serological methods (ELISA), and molecular techniques such as PCR and RT-PCR. These methods allow sensitive and specific detection of known pathogens. However, they are not suitable for the identification of unknown or highly genetically diverse viruses. In recent years, high-throughput sequencing (HTS) has provided new opportunities in plant virus diagnostics, as it enables the characterization of the complete virome of a sample without prior knowledge of the pathogens present. In particular, small RNA-based high-throughput sequencing plays an important role in plant virus diagnostics, allowing the detection of viruses and viroids without previous virus-specific information.

HTS-based virus diagnostics is especially important for improving the plant health safety of grapevine propagation and production, as it enables the detection of known, newly discovered, and latent viruses and viroids. Accordingly, the application of this approach may contribute to improving the health status of Hungarian vineyards and to reducing economic losses.

In the present study, Hungarian grapevine rootstock plantations and rootstock collections were investigated for viral and viroid infections, primarily using small RNA-based HTS. The specific objectives of the research were as follows:

- to determine the virome of Hungarian grapevine rootstock plantations and rootstock collections using small RNA-based HTS;
- to investigate potential alternative host plants of Grapevine Pinot gris virus (GPGV);
- to evaluate the possible occurrence of Grapevine virus T (GVT) in Hungary, the genetic diversity of its variants, and the sensitivity of HTS and RT-PCR for virus detection by reanalysis of HTS data from grapevine production and rootstock vineyards.

2. MATERIALS AND METHODS

Plant samples used in this study were collected from grapevine rootstock plantations and productive vineyards, as well as from rootstock collections, located in several wine regions of Hungary. During sampling, mainly symptomless plants that appeared healthy were selected, and different plant tissues were collected. For the investigation of GPGV, grapevine samples were collected together with weed plants growing in the surroundings of the vineyards. These weed samples showed virus-like symptoms. For the analysis of GVT, previously generated small RNA sequencing datasets were re-analysed.

Total RNA was isolated from woody and herbaceous plant samples using different extraction protocols adapted to the plant material. For small RNA-based HTS, RNA samples from individual plants were combined into plantation pools, each pool representing one vineyard. The small RNA fraction was isolated from these pools prior to library preparation. Small RNA libraries were prepared using a commercial library preparation kit following an optimized protocol. Sequencing was performed on an Illumina platform.

Bioinformatic analysis of the small RNA sequencing data was carried out using CLC Genomics Workbench software. After adapter removal and quality filtering, de novo contig assembly was performed. The assembled contigs were then aligned to reference genomes of plant viruses and viroids. The presence of viruses and viroids was evaluated based on several criteria. These included the presence of virus-specific contigs, normalized small RNA read counts, and the level of coverage of the viral genome.

Viruses and viroids identified by small RNA HTS were confirmed by reverse transcription PCR (RT-PCR). When necessary, positive PCR products were cloned and sequenced using Sanger sequencing. Phylogenetic analyses were performed using appropriate software tools to investigate the genetic relationships among virus isolates.

3. RESULTS AND DISCUSSION

The results of this study are presented and discussed below, following the order of the defined research objectives.

3.1 Virome analysis of grapevine rootstock plantations and rootstock collections using small RNA HTS

The viromes of Hungarian grapevine rootstock plantations and rootstock collections were investigated using small RNA-based HTS. The aim of this analysis was to obtain an overview of the virus and viroid composition of these plantations, with special focus on pathogens relevant to grapevine propagation material.

According to current regulations, most of the regulated viruses were not detected in the analysed samples. However, the presence of grapevine fleck virus (GFkV) was identified in two rootstock plantations and in the rootstock collections. Although GFkV may remain symptomless in many *Vitis vinifera* cultivars, it cannot be considered a latent virus in grapevine rootstocks and its presence is not permitted in certified propagation material. These results indicate that small RNA HTS is suitable for the simultaneous screening of regulated grapevine viruses. At the same time, in pooled samples representing whole plantations, low-titer infections may remain undetected and therefore require confirmation by targeted RT-PCR assays.

Among non-regulated viruses, several pathogens previously reported in Hungary were detected at high frequency. Grapevine rupestris stem pitting-associated virus (GRSPaV) was present in almost all analysed plantations, which is consistent with its known widespread and often latent occurrence in grapevine. Grapevine Pinot gris virus (GPGV) and grapevine Syrah virus 1 (GSyV-1) were also detected in multiple samples, showing variable genome coverage and small RNA read numbers, indicating heterogeneous distribution and different infection levels.

Among viroids, hop stunt viroid (HSVd) was the most frequently detected in all investigated plantations. Although viroid infections were mostly symptomless, their presence may negatively affect vine vitality and the quality of propagation material

in the long term. In addition, Australian grapevine viroid (AGVd) was detected in one rootstock collection. To our knowledge, this is the first report of AGVd in grapevine in Hungary. The application of small RNA-based HTS provided a comprehensive overview of viroid infections and enabled the detection of a viroid not previously reported in Hungary (AGVd).

Overall, the virome analysis of grapevine rootstock plantations showed that most propagation sources are free from economically important regulated viruses. However, non-regulated viruses and viroids are widely present. These results demonstrate that small RNA-based HTS is an effective tool for comprehensive virological surveys of grapevine rootstocks and can serve as a valuable complement to targeted RT-PCR-based diagnostic approaches.

3.2 Investigation of potential alternative hosts of Grapevine Pinot gris virus (GPGV)

To better understand the occurrence and possible transmission routes of GPGV, the study was extended to weed species present in the surroundings of grapevine plantations, in addition to grapevine samples. The aim was to investigate whether GPGV can infect non-Vitis hosts and whether these plants may contribute to virus persistence in vineyard environments.

In each investigated vineyard, grapevine samples were collected in parallel with weed sampling to confirm the presence of GPGV in the plantation. GPGV was detected in grapevine samples from all investigated vineyards using PCR-based methods. RT-PCR analyses revealed the presence of GPGV in several non-Vitis plant species, including annual and perennial herbaceous plants as well as woody species, providing new information on the potential alternative host range of the virus. Detection of GPGV was further examined by Northern blot analysis, which successfully confirmed the virus in two weed samples, specifically rose and blackberry.

Sequence analysis of GPGV variants showed that, in most investigated areas, virus variants detected in weed species were highly similar to those found in grapevine. In one of the five investigated vineyards, a heterogeneous distribution of virus variants was observed, likely related to the long-term lack of cultivation and the dense weed vegetation covering the grapevines.

These results indicate that certain weed species may serve as potential alternative hosts for GPGV and act as symptomless reservoirs contributing to virus persistence in vineyard surroundings. This is epidemiologically relevant, as surrounding vegetation may influence virus spread and long-term maintenance even when virus-free propagation material is used.

3.3 Occurrence and genetic diversity of Grapevine virus T (GVT) in Hungary

The presence of GVT in Hungary was investigated by re-analysing previously generated small RNA-based HTS datasets obtained from grapevine rootstock and production vineyards. At the time of sampling and initial data analysis, GVT was not yet known, therefore no targeted search for this virus had been performed. Its detection became possible through retrospective bioinformatic analysis.

During the re-analysis, GVT-related sequences were identified in only a limited number of HTS libraries. This indicates that small RNA-based HTS alone is not sufficient for the reliable detection of GVT. The low number of virus-derived small RNAs and the lack of virus-specific contigs suggest that GVT is present at low titer and shows a largely latent infection pattern, similar to other grapevine viruses that induce weak RNA silencing responses.

To confirm the presence of GVT and to investigate its actual occurrence in Hungary, targeted RT-PCR analyses were performed. Using RT-PCR, GVT was detected in several grapevine samples originating from both rootstock plantations and production vineyards. Importantly, some samples that were negative by HTS were positive by RT-PCR, clearly demonstrating the higher sensitivity of targeted molecular detection for this virus.

Sequence analysis of the detected GVT isolates revealed considerable genetic variability among the Hungarian samples. Several genetically distinct variant groups were identified, indicating that GVT is present in Hungary in multiple, genetically differentiated forms rather than as a single uniform variant population. This observed diversity is consistent with a long-term, unnoticed presence of the virus in grapevine plantations.

Overall, these results show that, in the case of GVT, small RNA-based HTS and RT-PCR should be interpreted together. While HTS can provide indications of virus presence and allow the analysis of genetic diversity, RT-PCR is essential for reliable detection and for assessing virus distribution. This highlights the importance of a combined diagnostic approach for viruses that are present at low titer and cause latent infections.

4. CONCLUSIONS AND RECOMMENDATIONS

Previous virological surveys of Hungarian grapevine plantations using small RNA-based HTS have demonstrated the importance of this method in plant virus diagnostics and provided a basis for targeted evaluation of the sanitary status of grapevine propagation material. Accordingly, in this study we investigated the virus and viroid infections of Hungarian grapevine rootstock plantations and rootstock collections, which is of major importance for the safety of grapevine propagation material.

Based on the examination of rootstock plantations and collections, it can be concluded that most regularly inspected rootstock plantations are free from officially regulated viruses, confirming the effectiveness of the current control system. In contrast, in rootstock collections where such regular inspections are not applied, regulated viruses and viroids were also detected. This supports the view that conventional diagnostic methods are suitable for eliminating pathogens that are critical for certification, but they do not provide a complete picture of the virological status of the plants.

The widespread occurrence of non-regulated viruses and viroids, particularly GRSPaV, GSyV-1, GPGV, as well as HSVd and GYSVd-1, indicates that these pathogens are endemic in Hungarian grapevine propagation systems. The first detection of AGVd in a Hungarian rootstock collection provides new information on grapevine virology in Hungary and highlights the need for further targeted investigations.

Differences observed between RT-PCR and small RNA-based HTS results, especially in the case of GRSPaV and GVT, indicate that reliable detection of certain viruses is only possible by combining different diagnostic approaches. The sensitivity of the methods is strongly influenced by the genetic variability of viruses and by the suitability of reference sequences used during bioinformatic analyses.

The investigation of GPGV confirmed that the virus can persist not only in grapevine but also in several non-Vitis plant species. Herbaceous and woody plants present in

the vineyard environment may play a role in the long-term persistence and spread of the virus. The increased genetic variability observed in vineyards that had not been cultivated for a long time suggests that local virus populations may undergo substantial genetic changes over time.

Reanalysis of previously generated small RNA HTS datasets combined with targeted RT-PCR assays confirmed the presence of GVT in Hungary. Although the virus occurs at a generally low frequency, locally higher infection levels were observed in some productive vineyards. Phylogenetic analyses showed that Hungarian GVT variants belong to multiple evolutionary lineages, suggesting that the virus may originate partly from imported propagation material and partly from long-established local virus populations.

Overall, our results demonstrate that assessment of the complete plant health status of grapevine propagation material requires consideration of non-regulated viruses and viroids as well. High-throughput sequencing represents an important complementary tool to conventional diagnostic methods; however, its reliable application requires appropriate standardization and validation. The gradual integration of HTS into diagnostic systems may contribute in the long term to the development of a more comprehensive and modern certification framework and to the reduction of plant health risks in grapevine production.

5. NEW SCIENTIFIC RESULTS

1. Using small RNA-based HTS, we determined the virome of 17 grapevine rootstock plantations and 2 grapevine rootstock collections in Hungary.
2. We identified the presence of Australian grapevine viroid (AGVd) in grapevine in Hungary for the first time.
3. The presence of Grapevine Pinot gris virus (GPGV) was detected for the first time in Hungary in common lambsquarters (*Chenopodium album*), and worldwide for the first time in seven additional plant species: common milkweed (*Asclepias syriaca*), blackberry (*Rubus* sp.), dog rose (*Rosa canina*), elderberry (*Sambucus* sp.), hawthorn (*Crataegus* sp.), tree of heaven (*Ailanthus* sp.), and ash (*Fraxinus* sp.).
4. By reanalysis of small RNA-based HTS data from Hungarian grapevine production vineyards and rootstock plantations, we confirmed the presence of Grapevine virus T (GVT) in Hungary for the first time.

6. PUBLICATIONS RELATED TO THE TOPIC OF THE DOCTORAL DISSERTATION

International peer-reviewed journal articles (Q1–Q2)

Demián, E., Jaksa-Czotter, N., Molnár, J., Tusnády, G. E., Kocsis, L., Várallyay, É. (2020). *Grapevine rootstocks can be a source of infection with non-regulated viruses.* **European Journal of Plant Pathology**, 156, 897–912.
(Scopus: Horticulture – Q1; Agronomy and Crop Science – Q2; Plant Science – Q2)

Demián, E., Jaksa-Czotter, N., Várallyay, É. (2022). *Grapevine Pinot Gris Virus Is Present in Different Non-Vitis Hosts.* **Plants**, 11(14), 1830.
(Scopus: Ecology – Q1; Ecology, Evolution, Behavior and Systematics – Q1; Plant Science – Q1)

Demián, E., Holczbauer, A., Nagyné Galbács, Zs., Jaksa-Czotter, N., Turcsán, M., Oláh, R., Várallyay, É. (2021). *Variable populations of Grapevine virus T are present in vineyards of Hungary.* **Viruses**, 13(6), 1119.
(Scopus: Infectious Diseases – Q1; Virology – Q2)

International peer-reviewed journal articles (Q3–Q4)

Jaksa-Czotter, N., Nagyné Galbács, Zs., Jahan, A., **Demián, E.**, Várallyay, É. (2024). *Viromes of plants determined by high-throughput sequencing of virus-derived siRNAs.* **Methods in Molecular Biology**, 2732, 179–198.
(Scopus: Genetics – Q4; Molecular Biology – Q4)

Czotter, N., Molnár, J., Pesti, R., **Demián, E.**, Baráth, D., Varga, T., Várallyay, É. (2018)
Use of siRNAs for diagnosis of viruses associated to woody plants in nurseries and stock collections. **Methods in Molecular Biology**, 1746, 115–130.
(Scopus: Genetics – Q3; Molecular Biology – Q3)

Book Chapters

Czotter, N., **Demián, E.**, Várallyay, É. (2019). *Vírusdiagnosztika kis RNS-ek nagy áteresztőképességű szekvenálásával.* In: Szabó, P. (szerk.) **Innováció a szőlőszaporításban**. Budapest, Magyarország: Doktoranduszok Országos Szövetsége (DOSZ), 60–65.

Demián, E., Turcsán, M., Jaksa-Czotter, N., Varga, T., Szénási, M., Kocsis, L., Oláh, R., Várallyay, É. (2019). *Szőlő alanyültetvények virológiai felmérése és a különböző patogénmentesítési eljárások hatékonyságának vizsgálata kis RNS-ek nagy áteresztőképességű szekvenálásával.* In: Szabó, P. (szerk.) **Innováció a szőlőszaporításban**. Budapest, Magyarország: DOSZ, 66–71.

Other publications

Demián, E., Várallyay, É. (2019). *A szőlő Pinot gris vírus Magyarországon.* **Kertészet és Szőlészet**, 68(28), 20–21.

Conference presentations and posters

Demián, E., Turcsán, M., Varga, T., Jaksa-Czotter, N., Molnár, J., Szénási, M., Tusnády, G. E., Oláh, R., Várallyay, É. (2019). *Viral diagnostics of rootstock plantations and the complex pathogen elimination methods of grapevine by small RNA NGS.* **Hungarian Molecular Life Sciences 2019**, Eger, Hungary. Poszter.

Demián, E., Czotter, N., Molnár, J., Tusnády, G. E., Várallyay, É. (2017) *Magyar alanyszőlő ültetvények vírusdiagnosztikája kis RNS-ek újgenerációs szekvenálásával.* **63. Növényvédelmi Tudományos Napok**, Budapest. Poszter.

Demián, E., Czotter, N., Czakó, K., Molnár, J., Tusnády, G. E., Várallyay, É. (2017). *Detection and molecular characterization of Hungarian strain of Grapevine Pinot gris Virus.* **Hungarian Molecular Life Sciences 2017**, Budapest. Konferencia-absztrakt.

Demián, E., Czotter, N., Várallyay, É. (2018). *Detection of Grapevine Pinot gris virus in different non-Vitis hosts in Hungary.* **19th Conference of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG)**, Proceedings, 24–25. Előadás.

Demián, E., Czotter, N., Várallyay, É. (2018). *Investigation of Grapevine Pinot gris virus presence in non-Vitis hosts in Hungary.* In: Bielen, A. et al. (szerk.) **Power of Viruses – Programme and Abstracts**. Zagreb, Horvátország: Croatian Microbiological Society, 76. Előadás.

Demián, E., Turcsán, M., Varga, T., Czotter, N., Szénási, M., Oláh, R., Várallyay, É. (2019). *Szőlő alanyültetvények és a komplex patogénmentesítési módszerek hatékonyságának vírusdiagnosztikája.* **XXIX. Keszthelyi Növényvédelmi Fórum**, Keszthely, Magyarország.
Előadás.

Demián, E., Jaksa-Czotter, N., Molnár, J., Kocsis, L., Tusnády, G. E., Várallyay, É. (2019).
A szőlő Pinot gris vírus hazai előfordulása szőlőalany-ültetvényeken és gyomnövényeken.
„A szőlő szaporítóanyag-előállítás és a szőlőtermesztés növényvédelmi kérdései" című regionális rendezvény, Keszthely, Magyarország. Előadás.

Demián, E., Kontra, L., Jaksa-Czotter, N., Lázár, J., Várallyay, É. (2019). *Vírusdiagnosztikával a szőlő furcsa tüneteinek nyomában.* In: Jakab, G. (szerk.) **Szőlészeti és borászati kutatások Pécsett, és kapcsolódásuk a hazai és külföldi irányvonalakhoz**. Pécs, Magyarország: PTE Szőlészeti és Borászati Kutatóintézet, 16. Előadás.

Demián, E., Czotter, N., Várallyay, É. (2018). *Grapevine Pinot gris vírus (GPGV), egy szőlővírus gyomnövényeken.* **XXVIII. Keszthelyi Növényvédelmi Fórum**, Keszthely, Magyarország. Előadás.

Demián, E., Turcsán, M., Varga, T., Czotter, N., Szénási, M., Oláh, R., Várallyay, É. (2018).
Szőlő alanyültetvények és a komplex patogénmentesítési módszerek hatékonyságának vírusdiagnosztikája. **MBK Napok**, Gödöllő, Magyarország. Előadás.

Demián, E., Czotter, N., Czakó, K., Várallyay, É. (2017). *A Grapevine Pinot gris vírus elterjedése szőlőültetvényeken és gyomokban.* **MBK Napok**, Gödöllő, Magyarország. Előadás.

Demián, E., Czotter, N., Várallyay, É. (2017). *Alkalmazható-e a kisRNS NGS új vírusgenom meghatározására Sanger-szekvenálás nélkül?* Tudományos előadás, Magyarország.