Final report of NKFIH-OTKA funded project K119783

Molecular characterization of function, importance and evolution of new emerging grapevine viruses by high throughput sequencing and molecular biology-based methods

In our project we used molecular base methods to get a closer look at virus infection in grapevine. To conduct these surveys first we optimised diagnostic methods, small RNA HTS, its validation with unrelated techniques together with the methods used for virus elimination.

Our published protocols which were optimised within the frame of this project:

i/improvement in RT-PCR and tissue culture techniques:

- Oláh, Róbert, Deák, Tamás, Mihály, Turcsán, Szénási, Márta, Bordé, Ádám and Szegedi, Ernő. (2017). Use of an intron containing grapevine gene as internal control for validation of cDNA synthesis in virus detection by RT-PCR. **European Journal of Plant Pathology**. 149. 1-6. 10.1007/s10658-017-1218-5. **IF:1,582**
- Szegedi, E., Deák, T., Turcsán, M., Szénási, M., Bordé, Á., and Oláh, R. (2018). Evaluation of intron containing potential reference gene-specific primers to validate grapevine nucleic acid samples prepared for conventional PCR and RT-PCR. **Vitis**, 57, 69-73. **IF:1,532**
- Robert Oláh (2017) The use of activated charcoal in grapevine tissue culture. **Vitis** 56, 161–171 DOI: 10.5073/vitis.2017.56.161-171 **IF:1,532**

ii/ small RNA HTS as a virus diagnostic method

Small RNA HTS is based on Illumina sequencing and bioinformatics analysis of virus-derived small RNAs in the host. We described the whole protocol for this challenging technique step by step with notes, from RNA extraction till bioinformatics analysis, in order to ensure success for every user:

- 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**. In: Viral Metagenomics: Methods and Protocols.** (Pantaleo, V. and Chiumenti, M., eds.). New York, NY: Springer New York, pp. 115-130. doi.org/10.1007/978-1-4939-7683-6_9.

To try how efficiently this method can be used for different varieties we used this method also for fruit trees and with its help we identified Little cherry-1 virus in apricot, as a new host:

- Baráth, D.; Jaksa-Czotter, N.; Molnár, J.; Varga, T.; Balássy, J.; Szabó, L.K.; Kirilla, Z.; Tusnády, G.E.; Preininger, É.; Várallyay, É. Small RNA NGS Revealed the Presence of Cherry Virus A and Little Cherry Virus 1 on Apricots in Hungary. **Viruses** 2018, 10, 318. doi.org/10.3390/v10060318 **IF:3,811. cited 4** times till 15.12.2020.

In our previous OTKA project we finished a work in which not only gene-expression changes of virus infected tobacco and tomato were identified by high-throughput microarray or RNAseq, but leaf temperature and chlorophyll fluorescents parameters of virus infected hosts were measured. We were able to link the fundamental differences in the host gene-expression patterns of acute and persistent virus infections to differentially altered host physiology even in the early phase of the infection, when visible symptoms slightly appeared. High-throughput sequencing methods lead to the discovery of new viruses each day, including several latent ones which has been coexisted or even coevolved as persistent infection with their hosts. Type of infection which viruses cause can vary from host to host and must be determined in order to

show their actual importance. Economically important agronomic losses are results mainly of acute infections why knowledge about basic differences of acute and persistent infection can give a help during risk assessment of these recently described viruses. Although this work based on the previous OTKA its finalization was done by this project. We not only compared gene-expression, but physiological changes at the early time of the infection which could have used in the future in monitoring systems to reveal early status of viral infections.

- Réka Pesti, Levente Kontra, Kenny Paul, Imre Vass, Tibor Csorba, Zoltán Havelda and Éva Várallyay* (2019) Differential gene expression and physiological changes during acute or persistent plant virus interactions may contribute to viral symptom differences**. PLoS ONE** 14(5) https://doi.org/10.1371/journal.pone.0216618. **IF:2,74. cited 5** times till 15.12.2020.
-

This work served as a base of the **PhD thesis of Pesti Reka** who defended her thesis in November 2020.

The above-mentioned technical advances made us possible to investigate the aimed research. To make easier to follow progress on the planned topics numbers refer to the chapter number in the project proposal.

1. **Molecular characterization of GPGV and GSyV1**

At the beginning of the project, we surveyed vineyards at different location in Hungary via small RNA HTS. Analysis of the sRNA sequence dataset obtained using our bioinformatics pipeline enabled us to describe viruses never before reported from our country (Grapevine Syrah virus1 and Grapevine Pinot Gris virus, Grapevine Satellite virus). Beside these new descriptions we analysed our samples for the most widespread viruses with RT-PCR (using published diagnostic primers) and sRNA HTS in parallel. In most cases our results could be verified, but we also found contradiction in several cases. Virus presence did not correlate with the age of the plantation, moreover phylogenetic analysis of the identified virus isolates suggests that infections are mostly caused by the use of infected propagating material. Our results, validated by other molecular methods, raised further questions to be answered before this method can be introduced as a routine, reliable test for grapevine virus diagnostics.
This work was published in Frontiers in Microbiology:

- Nikoletta Czotter, Janos Molnar, Emese Szabó, Emese Demian, Levente Kontra, Ivett Baksa, Gyorgy Szittya, Laszlo Kocsis, Tamas Deak, Gyorgy Bisztray, Gabor E. Tusnady, Jozsef Burgyan and **Eva Varallyay*** (2018) NGS of virus-derived small RNAs as a diagnostic method used to determine viromes of Hungarian vineyards. **Frontiers in Microbiology** 9, 122 (2018) doi.org/10.3389/fmicb.2018.00122 **IF: 4,23. cited 20** times till 15.12.2020.

This work served as a base of the **PhD thesis of Nikoletta Czotter** who defended her PhD thesis in November 2019.

In this part of the project, we also aimed to investigate the possible viral silencing suppressor role of different ORFs of GPGV and GSyV1. To do that we have cloned and sequenced different isolates of GPGV and GSyV1. The resulted sequences were deposited into GenBank. GSyV1 from different individuals of the same plantation showed high variability what supports the idea that GSyV1 population in Central Europe is more diverse than the North American ones. According to GPGV CP sequences our isolates showed slight variation, but grouped distantly and together with isolates of different geographical origins, which supports the possibility that GPGV spread from Eastern Europe to Italy, and from Europe to other parts of the world.

We have cloned the putative MP of GPGV into binary vector and made preliminary transient assays to test if it has any silencing suppressor activity. According to this preliminary test we found that further optimisation would need for the reliable conclusion. Because of some personnel problems we left this part of the experiments to the last year of the project, but finally we could not finish that. We still have to answer if MP of GPGV can act as a viral suppressor and can it be that difference between the virulent and avirulent strains.

2. **Investigation of sensitivity of Hungarian varieties**

We have cloned the full GPGV, made infectious transcript of it, but were unable to infect grapevine with that transcript, why we cannot use this way to find out the possible different susceptibility of Hungarian strains for that virus.

As we suspected that this effort could fail, we started to compare different methods for virus elimination. Analysis of small RNA HTS results from mother plants, meristematic cultures (ME) and somatic embryogenesis (SE)lines revealed that somatic embryogenesis work fine for all of the presented viruses, while the traditional meristematic cell cultures was not effective against GPGV and GRSPaV. Our results showed for the first time that elimination of all viruses in the mother plants with SE was effective, including grapevine rupestris vein feathering virus (GRVFV), grapevine Syrah virus 1 (GSyV-1), Grapevine virus T (GVT) and grapevine Pinot gris virus (GPGV). This study also confirms previous studies showing that SE is a possible strategy for the elimination of GFkV, GRSPaV, HSVd, and GYSVd-1. Our results demonstrated that the efficacy of virus elimination via SE was relatively high while purging of viroids was lower. Our work demonstrated that the efficiency of SE is comparable to the technically difficult ME technique, and offers a more effective strategy to meet the high demand of the production of virus-free grapevine with its use in the future.
This work was accepted for publication (14.12.2020) at MDPI Plants:

- Mihaly Turcsan, Emese Demian, Tunde Varga, Nikoletta Jaksa-Czotter, Erno Szegedi, Robert Olah, Eva Varallyay * (2020): Title: HTS-based monitoring of the efficiency of somatic embryogenesis and meristem cultures used for virus elimination in grapevine. **MDPI Plants**: **IF: 2,762.**

We have found problems during elimination of GRSPaV, and thought that in-situ hybridization of GPGV and GRSPaV containing shoot tips could show that are these viruses entering into the meristem, or not. We planned these experiments and we already embedded infected shoot tips for that. Unfortunately, we could not finish that part of the research, but plan to conduct that experiments in the future.

3. **Investigation of molecular evolution processes in fleck type viruses.**

As planned, we collected samples in the neglected vineyards at Mogyoród from different plant species. For our surprise we have found GPGV not only in grapevine but in some of the investigated weeds. We have cloned and compared variable region of GPGV from weeds and grapevine originating not only from this place. Beside grapevine presence of GPGV was detected on 5 different non-Vitis hosts: *Chenopodium* sp., *Asclepias syriaca*, *Rosa* sp, *Rubus* sp. and *Fraxinus* sp. by RT-PCR, but only when cDNA was amplified by virus specific probe, indicating a lower virus level comparing to grapevine. In case of *Rosa* and *Rubus* we could support this finding by Northern blot detection of the virus. The presence of GPGV in vineyard's neighbouring woody or perennial hosts suggests that it could be true that GPGV is endemic in Eastern Europe and spread from here to the other part of Europe. Phylogenetical analysis of the replicase fragment and MP/CP region of the samples showed that GPGV variants were clustered according to the vineyards and not according to the hosts. We show

these results at Keszthely at **Növényvédelmi Fórum** and at 19th ICVG meeting as an oral presentation.

- Emese Demián, Nikoletta Czotter and Éva Várallyay (2018) Detection of Grapevine Pinot gris Virus in different non-Vitis hosts in Hungary. **19th Conference of the ICVG, Santiago of Chile**, 9-12 of April, 2018.

Since that we prepared small RNA sequencing libraries from these "weeds", sequenced them at Illumina platform and analysed them with bioinformatics methods, which supported the original findings that yes, GPGV is present in them. We plan to finish our manuscript describing these results in the near future.

Fleck virus evolution was investigated in a vineyard at Pecs. small RNA HTS and Sanger sequencing of GRVFV at this plot showed high variation why we chose this place for evolution studies. Individuals at this plantation were investigated for the presence of different fleck type viruses, we cloned and Sanger sequenced them and compared their sequences and found that recombination events between closely familiar viruses took place when they coinfected the same plant. We have compared different GRVFV isolates at this particular plantation. Phylogenetic comparison of the amplified virus sequences showed that recombination events happened not only at the plantation but even at an individual level. This work was summarized as an **MSc thesis of Dino Muratovic**, agricultural biotechnologist at SZIU.

4. **Survey of Hungarian rootstock plantations**

Our previous survey of vineyards in Hungary suggested that viral infection originates from infected propagation material. To investigate whether rootstocks can be a source of virus infections, we surveyed seventeen certified rootstock plantations and two rootstock collections in Hungary to determine the virome by high-throughput sequencing of small RNAs. The presence of the viruses was also tested by RT-PCR. The results showed that viruses whose presence is routinely checked were almost absent in rootstock vineyards but were present in rootstock genotype collections. Moreover, first the time in Hungary, we detected the presence of Australian grapevine viroid in the rootstock genotype collection at Pecs. In contrast, viruses that are not regulated or not routinely tested, namely, grapevine rupestris stem-pitting-associated virus, grapevine Syrah virus-1 and grapevine Pinot gris virus, were detected in almost all locations in most of the varieties. The presence and absence of infected rootstock genotypes in the same vineyard together with phylogenetic analysis suggested that viral infections originated from infected propagation material. Moreover, we found the symptomatic variant of grapevine Pinot gris virus in several rootstock vineyards without symptoms, suggesting the possibility for leaf mottling and deformation disease symptoms to manifest on susceptible cultivars following grafting onto these rootstocks.

Our results were published at European Journal of Plant Pathology:

- Demian, E., Jaksa-Czotter, N., Molnar, J., Tusnady, G. E., Kocsis, L. and **Varallyay, E.*** (2020) Grapevine rootstocks can be a source of infection with non-regulated viruses. European Journal of Plant Pathology doi.org/10.1007/s10658-020-01942-w. **IF:1,774**

5. **Identification of the virus causing Grapevine line pattern disease symptoms.**

Grapevine line pattern is one of these diseases has been reported only from Hungary. Its first report dates back to 1987 to the 9th IVG meeting where Janos Lehoczky presented GLPV in a talk. Since that, itwas thought as a possible member of the Ilarvirus genus of Bromoviridae. During his enthusiastic work, Prof Lehoczky established a pathologic garden where he collected

all of the interesting grapevine viruses. GLPV was maintained on an interspecific hybrid Baco 22A, and although the executive administration of the viticulture research institute, owing this plot, changed several times, that vine, containing GLPV, is still exist there. In our work we sampled the original host, the Baco 22A, growing in the pathogen garden at Kecskemét-Katonatelep in order to identify the line pattern causing agent. Using small RNA HTS we identified a possible anulavirus. Primers designed according to the assembled reads were used for RT-PCR. Amplified product of both RNA 1, 2 and 3 were produced, cloned and sequenced by traditional Sanger sequencing. Phylogenetical analysis of the sequences of the amplified product showed that the virus present in Baco 22A belongs to Anulavirus genus. The gained sequence information makes us possible to investigate the presence of this virus at other geographical locations even in latent form.
Our results were published at MDPI Viruses:

- Elbeaino, T.; Kontra, L.; Demian, E.; Jaksa-Czotter, N.; Slimen, A.B.; Fabian, R.; Lazar, J.; Tamisier, L.; Digiaro, M.; Massart, S.; **Varallyay, E.* (2020)** Complete Sequence, Genome Organization and Molecular Detection of Grapevine Line Pattern Virus, a New Putative Anulavirus Infecting Grapevine. ***Viruses***, *12*, 602. doi.org/10.3390/v12060602. **IF:3,811. ones** till 15.12.2020.

Some experiments were conducted by **Richard Fabian**, who included that result into his **MSc thesis** as a Plant protection engineer at SZIU, and defended his thesis in 2020.

HTS based virus description revolutionized in the last years. This is why there are several new viruses which presence were not known during our surveys.
One of these viruses is grapevine virus T (GVT), which presence is turned out to be very widespread in Slovakia and Czech Republic. In order to find out if it is present in Hungary and if yes, how widespread it is we reanalysed our small RNA libraries prepared from vineyards looking for the presence of GVT, a virus which has not been described when we made the original analysis and have found it at several vineyard. Our student **Holczbauer Aliz** (biologist ELTE) has defended her **BSc thesis** describing this work. Since that we have not only reanalysed our small RNS libraries originating from rootstock plantation for the same reason, but have successfully validated its presence with an independent RT-PCR method. Sequences of different strains were deposited into GenBank. Currently we are working on the manuscript describing our results.

During our field surveys we have found a plantation showing typical symptoms of grapevine red bloch associated virus (GRBaV), not reported from Hungary and even from Europe before. To investigate the presence of the virus we have prepared small RNA sequencing libraries from plants showing red bloch and leaf roll like symptoms. Results of this survey in what we could not verify the presence of GRBaV was presented at Keszthely at Növényvédelmi Fórum in January 2020 and served as a base for the **MSc thesis of Dana Khrais**. The manuscript describing our finding were accepted in Georgikon for Agriculture and will be published in the next issue.

This project made possible for **Emese Demian** to carry out her **PhD work**. She is on a maternity leave now. Her PhD thesis is not ready yet but she plans to finish next year and would also acknowledge this project.

Last, but not least I myself (**Éva Várallyay**) have finished (, but not defended yet, currently at the reviewers) my **Doctoral thesis**, and included the results what I got by the funds of this project.