Thesis Summary of the PhD Dissertation

VIRUS DIAGNOSTICS OF WEEDS ON CROP FIELDS AND INVESTIGATION OF THEIR ROLE AS VIRUS RESERVOIR

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1. INTRODUCTION, AIM AND OBJECTIVES OF THE DISSERTATION

Weeds contribute significantly to yield loss and reduction in yield quality in our agricultural dispensation, competing with the crop for essential resources like light, water and nutrients. Also, crop plants often grow side by side with weeds and wild plants. These plants constitute potential reservoirs for viruses that may spread into cultivated crops, thereby leading to epidemics or the emergence of novel viruses (Elena et al., 2014). Spread from reservoirs into a new environment, establishing productive infections and effective between-host transmission are necessary steps for virus emergence to occur. Most plant viruses are transmitted by insect vectors, which may increase the possibilities for virus transmission across landscapes and the distances over which viruses can be transmitted (Ng and Falk, 2006).

Climatic variations could contribute to the successful spread of newly introduced viruses or their vectors, and the presence of these organisms in new environments that were previously unfavorable for them. Global climate change is one of the main factors for the increased economic impact of aphids in temperate regions, where these aphids can acclimate to new environmental conditions rather quickly (Hullé et al., 2010, Forsius et al., 2013). Non-native plants and insects may also spread out their geographic ranges and hereby securing communities where they were previously not present. These species might constitute reservoir hosts or vectors of plant viruses capable of causing epidemics in nearby crops (Canto et al., 2009).

Also, plant viruses could spill over in both directions between weed plants and crops with potential adverse effects in both managed and natural ecosystems. Spillover of viruses onto cultivated plants usually occurs at the border of plant ecosystems (Elena, 2011; Stobbe and Roossinck, 2016). In most cases, the virus cannot adapt to further transmission in the organism of the new host, or its titers in plants become insignificant (low intensity of virus reproduction). However, sometimes "introduced" viruses can adapt to further transmission in the new host, leading to new emerging viral infections (Alexander et al., 2014).

Plant virus detection over the years centered on economically important crops, but to effectively manage plant viral infections associated with weeds, research has revealed the need to equally investigate surrounding weeds of crop fields so that strategic management and control practices are devised to manage infestations in crop fields. Plant virus diagnosis is crucial for developing

effective and sustainable crop management systems (Biswas et al., 2016), especially considering that several biotic and abiotic factors also produce virus-like symptoms in plants (Yadav and Khurana, 2016). The use of appropriate integrated control strategies upon virus identification will prove effective in mitigating the spread of the virus, thereby reducing further crop damage and yield loss. The other aspect of integrated management approaches is to prevent the dissemination of virus causing diseases through their different modes (López et al., 2009). The methods of detection and identification of viruses developed fall into serological techniques, molecular methods, microscopical and physical observations (Biswas et al., 2016).

High-throughput sequencing (HTS) is a rapidly evolving technique, delivering novel opportunities for diagnosis and epidemiology. This technique enables the sequencing of millions of DNA molecules in a short time, which facilitates the detection of the most viral pathogens in the sample (Elbeaino et al., 2018). Due to the possibility of sequencing millions of nucleotide sequences, it could uncover a global spectrum of occurring strains or species of pathogens. The multiplication of pathogens in plant cells could affect the health condition whether we diagnose or fail to diagnose pathogens (Elbeaino et al., 2018).

During plant virus infections, the RNAi-based defense mechanism of the plant is activated. During this mechanism, small interfering RNAs (siRNA) having indistinguishable sequences to the infecting viruses are produced consequently, these siRNAs could be isolated to investigate virus present (Nicaise, 2014; Pooggin, 2018). When an active plant virus is present in a plant cell, double-stranded RNAs (dsRNAs) are formed. These dsRNAs are cleaved by DICER enzymes into siRNAs. One strand of these siRNAs is incorporated into an RNA Induced Silencing Complex (RISC) which gives specificity and locates all RNAs which have sequence complementarity to the bound siRNAs, and will target the virus, as it was produced from the presenting plant virus (Pooggin, 2016; Fang and Qi, 2016).

The genome of the specific target RNA (in this case, the virus itself) could be cleaved and its activity blocked in the RISC. If this mechanism works efficiently, low amount of intact virus would be present in the plant, but high amount of virus specific siRNAs would be present. These virus specific siRNAs can be isolated and used in plant virus diagnosis to investigate the virus present and active in the plant (Figure 1). Small RNA high-throughput sequencing (sRNA HTS) together with its bioinformatics analysis of investigated plant samples brings an exceptional avenues to

uncover the occurrence of any virus present in the sample, including new ones never described or identified (Kreuze et al.,2009).

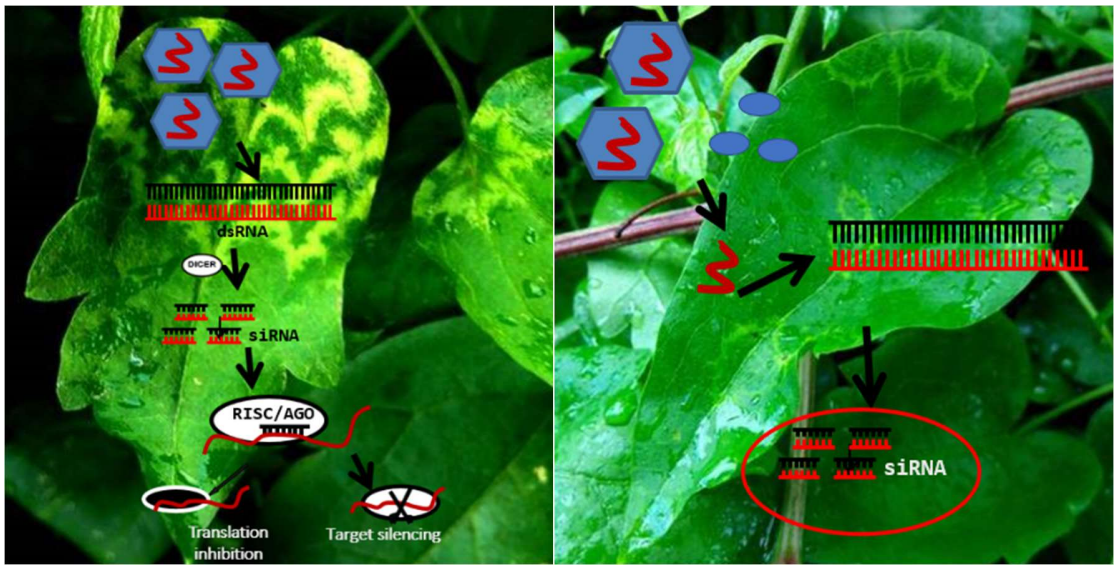


Figure 1. RNA Interference. The siRNAs isolated from the infected plant can be used to diagnose the plant virus present (Photo by Pal Salamon).

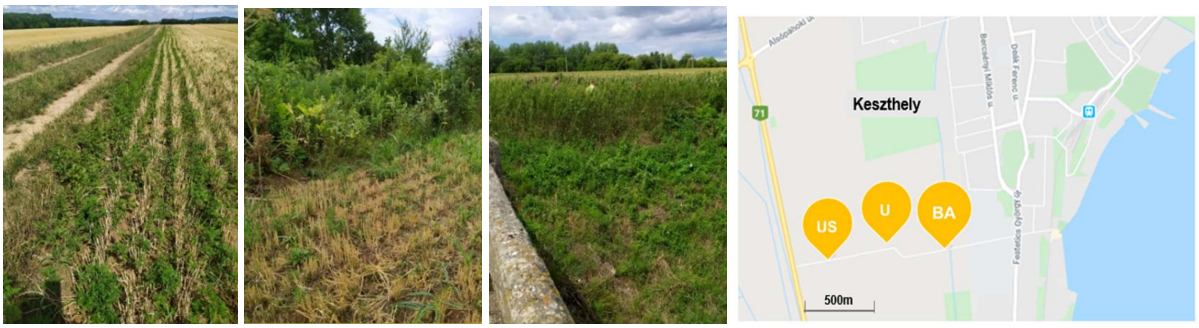
The aim of the research is to investigate virus infection in monocotyledonous weeds present in crop fields and to uncover their role in epidemics. Virus diagnostics was carried out not only by using RT-PCR based methods, but also small RNA High throughput sequencing (sRNA HTS), which is able to disclose presenting plant viruses in the investigated sample. The research was achieved by undertaking the following objectives:

a) To conduct a survey of monocotyledonous weeds showing virus-like symptoms on agricultural experimental fields in Keszthely.
b) Investigation of potential plant virus reservoir role played by weeds of the agricultural study location. In our previous project of Pasztor et al. (2020) surveying two millet (*Panicum milliaceum*) populations, wheat streak mosaic virus (WSMV), barley yellow striate mosaic virus (BYSMV) and barley virus G (BVG) were detected in millet samples of the experimental fields. This research further investigates the possible virus reservoir role and virus persistence in millet samples of these fields using small sRNA HTS.
c) To validate the outcome or results of the sRNA HTS obtained using independent RT-PCR based method.
d) To conduct phylogenetic analysis of the presenting viral strains, and to assess diversity of virus strains.

2. MATERIAL AND METHODS

2.1. Sample Collection

Sample collection was carried out in August 2021 in Keszthely as the experimental location. Symptomatic weed plants showing virus-specific symptoms were sampled at three different fields, US, U and BA to conduct deeper studies on the possible virus infections and virus reservoir role of sampled weeds from these experimental fields (Figure 2). In 2019, 2020 and 2021 wheat, corn and wheat were grown on the US field whilst potato, corn and corn were cultivated on the BA field respectively. No intense cultivation had taken place at the time of sampling at the experimental location of U. At US, where wheat was just harvested, *P. milliaceum*, *E. crus-galli*, *S. viridis* and *C. dactylon* were randomly sampled. At U, *S. halepense* weed plants were sampled and at BA, *P. milliaceum*, *E. crus-galli*, *S. viridis* and maize plants were randomly sampled. *P. milliaceum* was grown as a weed.



US **U** **BA**

Figure 2. Photos (Agyemang) and a map (Google) about the sample collection areas of Keszthely, Hungary **US**: "Újmajor susnyás", **U**: "Újmajor" and **BA**: "Büdös árok".

2.2. RNA Extraction

Total nucleic acid extraction was carried out from frozen leaves sample using a phenol-chloroform method (Pasztor et al.,2020). The homogenization of the frozen plant material was done in an ice-cold mortar with 650 µL of extraction buffer (100 mM glycine, pH 9.0, 100 mM NaCl, 10 mM EDTA, 2% SDS, and 1% sodium lauroylsarcosine), followed by mixing with an equal volume of phenol and centrifuged for 5 min. Equal volumes of phenol, chloroform, and isoamyl-alcohol (25:24:1) were then added to the aqueous phase and centrifuged for 5 min. The obtained upper phase was supplemented with an equal volume of chloroform:isoamyl-alcohol (24:1) and

centrifuged for 5 min. This upper phase was precipitated, and washed with 70% ethanol. The resulting pellet was then centrifuged, dried and re-suspended in sterile water. The derived total nucleic acid extracts were kept in storage at −70 °C until used.

2.3. Small RNA Library Preparation, Sequencing and Bioinformatic analysis

RNA pools were made from individiduals of the same species and also from two different species of the same location. This was to ensure enough quality data and also enable the identification of any virus present in any of the individual samples. Using a polyacrylamide gel, sRNA fractions of the pools were separated, and purified using an in-house updated protocol (Jaksa-Czotter et al., 2024) centered on the TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA, USA). sRNA libraries were sequenced utilizing a single index on a HiScanSQ by UD-Genomed (Debrecen, Hungary) (50 bp, single-end sequencing). FASTQ files of the sequenced libraries were then subjected to bioinformatic analysis using the CLC Genomic workbench which helps to reveal all presenting plant viruses in the investigated samples.

2.4. Validation of the sRNA HTS results using RT-PCR

Complementary DNA (cDNA) synthesis of the RNA pools were conducted using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). With the use of Q5 DNA Polymerase (New England Biolabs, Ipswich, MA, UK), RT-PCR was conducted where primers amplify viral parts through the used annealing temperatures and cycling parameters set. GeneJET Gel Extraction Kit (Thermo Fisher Scientific) was employed in purifying the PCR products, followed by cloning into GeneJET (Thermo Fisher Scientific) and then, the clones were Sanger sequenced through a service order.

2.5. Phylogenetic Analysis

From the workbench, multiple sequence alignments were conducted using Geneious prime and MUSCLE algorithm. The evolutionary history was inferred using the Jukes-Cantor model and the Neighbour-joining method. The trees were constructed using the best fit model for each alignment, and 1000 boot-strap replicates. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. For the outgroups, the closest relative of the virus was referred to in the tree's legend.

3. RESULTS

3.1. Field Assessment, visual inspection and Weed Surveys

The weed samples collected from the study fields of US, U and BA displayed a diverse array of symptoms characteristic of virus infection. Symptoms ranged from mosaics, leaf deformations, tip necrosis, reddish brown colorations, mottling, chlorosis, purple discoloration and spots of leaves.

Újmajor susnyás		Symptoms
millet	M1	Ldef, M, Chl
	M2	M, Ldef
	M3	M, Ldef, P: Purple
	M4	Mosaic symptoms
	M5	Mosaic, Mild chlorosis
	M6	Mosaic, deformation
	M7	M, Chl, N, Stu, Ldef
	M8	M, Chl
	M9	M, Chl, Ldef
	M10	M, Chl, TN
	M11	M, TN
Echinocloa crus-galli	E1	Ldef, mild mosaic symptoms
	E2	Ldef, stunting
	E3	Ldef, Chl, stunting
	E4	Ldef, MM, Chl, Stu
	E5	Ldef, Chl, Necrosis, stu, M
	E6	Ldef, Stu, Chl, M
	E7	Chl,M,Ldef
	E8	Ldef,Stu,N, Chl,M
Setaria viridis	S1	M, Ldef, N
	S2	vein necrosis,Ldef, Purple coloration
	S3	M, Ldef, P, Chl
	S4	Ldef, Chl, P
	S5	M, N, TN, Ldef
	S6	Ldef, P, TN, Chl
Cynodon dactylon	C1	Ldef, Mosaic, Mottle
	C2	Ldef, M, Mo, Chl, TN
	C3	Ldef, M, Mo, Chl, TN
	C4	Ldef, MM, Mo
	C5	Mild Mosaic, TN

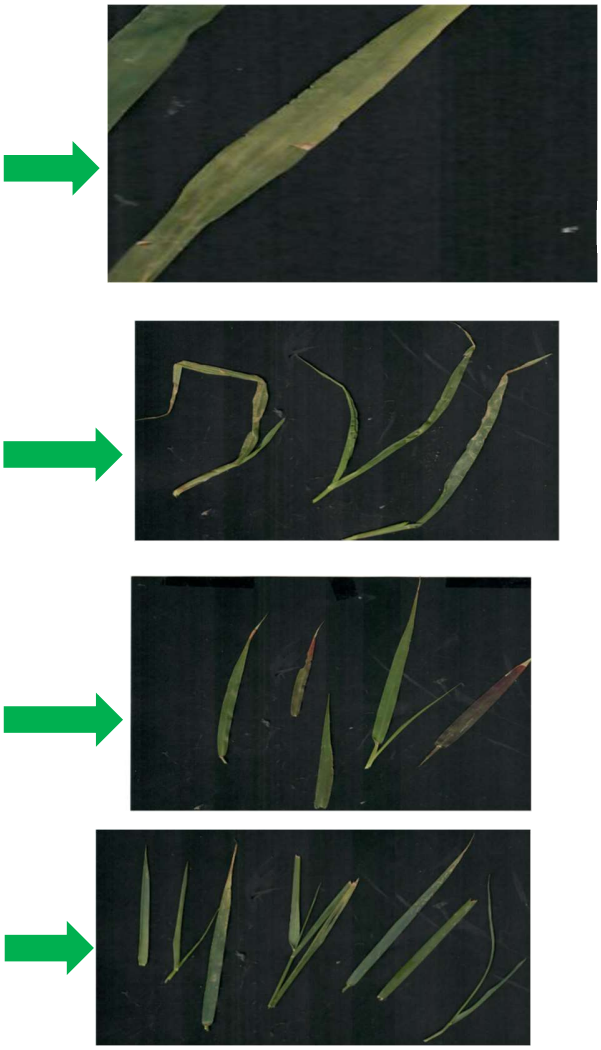


Figure 3. Samples collected from "Újmajor susnyás" (US) with their associated viral symptoms Ldef: leaf deformations, M: Mosaic symptoms, Chl: Chlorosis, P: Purple colorations, N: Necrosis, Stu: Stunting, TN: Tip necrosis, MM: Mild mosaic and Mo: Mottling.

In the experimental location of US, samples of millet (*P. milleaceum*) collected showed symptoms such as leaf deformations, mosaic, chlorosis, necrosis, stunting of the plant, tip necrosis and purple colorations. *E. crus-galli* weed samples were having mild mosaic symptoms, leaf deformations, necrosis, stunting and chlorosis. The samples of *S. viridis* weeds exhibited purple coloration, vein and tip necrosis, leaf deformations and mosaic whilst samples of *C. dactylon* weeds had mottling, leaf deformations, mosaic, chlorosis and tip necrosis (Figure 3).

In location U, where weed samples of *S. halepense* were collected, samples revealed stunting of the plant, leaf deformations, mosaic, tip necrosis and some minor purple coloration (Figure 4).

Újmajor		Symptoms
Sorgum halepense	H1	M, Ldef, TN
	H2	M, P
	H3	M, Stu, Ldef
	H4	Ldef, M, Stu, P
	H5	M, Stu, P

Figure 4. Samples collected from "Újmajor" (U) with their associated viral symptoms M: Mosaic symptoms, Ldef: leaf deformations, TN: Tip necrosis, P: Purple colorations and Stu: Stunting.

The samples of *P. milleaceum* from BA also indicated symptoms of chlorosis, mosaic, leaf deformation, stunting of the plant, necrosis, mottling and some pale purple colorations. Samples of *E. crus-galli* from this study location exhibited mosaic, chlorosis, leaf deformations and plants were stunted whilst *S. viridis* weeds samples of BA were having mosaic symptoms, tip necrosis, chlorosis and leaf deformations. Interestingly chlorotic leaves, some leaf deformations, mosaic and stunting were also observed in maize plants that were sampled from BA (Figure 5).

Büdös árok		Symptoms
millet	M2/1	M, Chl, Ldef
	M2/2	Stu, Chl, M, Ldef
	M2/3	Chl, N, Stu, Ldef
	M2/4	Stu, Chl, M
	M2/5	Chl, Ldef, M, N
	M2/6	Stu, Chl, M, TN
	M2/7	Chl, M, Ldef, P, N, Stu
	M2/8	M, Ldef
	M2/9	Stu, M, N
	M2/10	M, Chl, N, Mo
Echinocloa crus-galli	E2/1	Stu, Ldef, M
	E2/2	Ldef, Stu, Chl
	E2/3	M, Ldef
Setaria viridis	S2/1	Mosaic symptoms
	S2/2	M, Ldef, TN, Chl
	S2/3	Mosaic symptoms
maize	Ma1	Chl, M, Ldef
	Ma2	M, Stu, Chl
	Ma3	Chl, M, Stu
	Ma4	Stunning
	Ma5	Chl, Stu

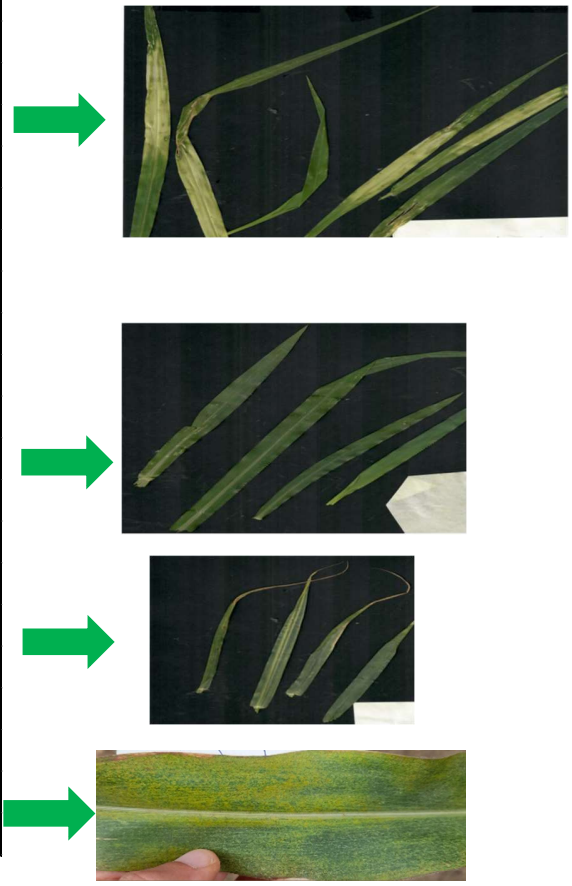


Figure 5. Samples collected from "Büdös árok" (BA) with their associated viral symptoms M: Mosaic symptoms, Chl: Chlorosis, Ldef: leaf deformations, Stu: Stunting, N: Necrosis, P: Purple colorations, Mo: Mottling and TN: Tip necrosis.

3.2. Results of the sRNA HTS study of the weed populations of the experimental locations.

The results of small RNAs high throughput sequencing revealed the viruses that were present in the investigated weed samples. The virus hits revealed the presence of WSMV, BYSMV, BVG, ApGlV1 and LDV1. After the 18–38 million sequenced reads were trimmed and quality controlled, 17–37 million redundant reads were maintained, which correspond to 1,5–4,2 million small RNA sequences (Table 1). Using the obtained non-redundant reads, 3103-20797 contigs could be assembled, and BLAST-ed to the reference genomes of known plant-infecting viruses.

Table 1. Statistics of the sequenced small RNA libraries of the investigated samples

Library code	Sequenced reads	Trimmed reads all (containing redundants)	Non-redundant reads	Number of contigs
1_M_US	21 453 088	21 273 768	3,351,469	11 377
2_ECG_US	23 965 784	23 623 757	2,497,935	12 366
3_SVCD_US	**38 236 832**	**37 392 543**	**4,198,026**	**20 797**
4_SH_U	**18 685 311**	**17 500 902**	**1,553,870**	3 586
5_M_BA	20 882 517	20 538 898	3,234,446	13 578
6_ECGSV_BA	32 030 185	31 197 132	2,408,431	8 461
7_Ma_BA	21 736 705	21 232 382	1,934,981	**3 103**

The contig hits with acceptable E-value (lower than 10^{-5}) revealed the presence of five viruses: WSMV, BYSMV, BVG, Aphis glycine virus 1 (ApGlV1) and Ljubljana dicistrovirus 1 (LDV1) (both in the same library: 7_Ma_BA) were found (Table 2).

Table 2. Bioinformatic results indicating viruses present in the investigated samples

Sample libraries	**Plant viruses detected or identified in the study**				
	WSMV	BYSMV	BVG	ApGlV1	LDV1
1-M-US	+				
2-ECG-US					
3-SVCD-US	+	+	+	+	+
4-SH-U					
5-M-BA	+	+		+	+
6-ECGSV-BA	+		+		+
7-Ma-BA				+	+

3.3. Validation of virus presence with RT-PCR

The validation of results of the sRNA HTS was conducted using an independent RT-PCR method. For the amplification, primers of Pasztor et al. (2020) study (in the case of WSMV, BYSMV and BVG) and newly designed ones (in the case of ApGlV1 and LDV1) were used. The results of the sRNA HTS were successfully validated. Moreover, all of the viruses were detected in additional libraries: WSMV was found in 2_ECG_US and 3_SVCD_US; BYSMV was also detected in 3_SVCD_US; BVG in 1_M_US, 2_ECG_US and 5_M_BA; ApGlV1 was detected in 4_SH_U and 6_ECGSV_BA; and LDV1 was identified in 3_SVCD_US, 5_M_BA and 6_ECGSV_BA according to RT-PCR results (Figure 6).

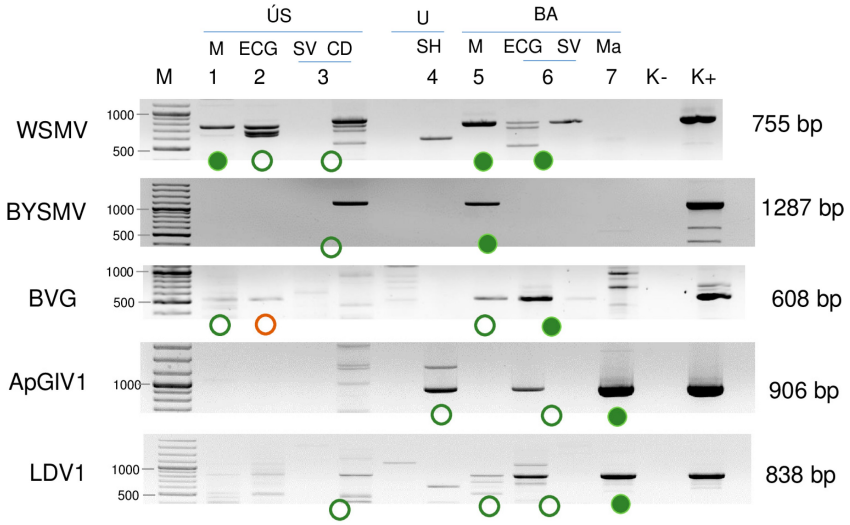


Figure 6. Virus diagnostics using RT-PCR and virus-specific primers. Circles indicate positive results, green when Sanger sequencing confirmed the presence of the virus, and red when Sanger sequencing did not confirm the RT-PCR result. Each library is represented only by one circle. Filled circled shows cases with the same sRNA HTS result. K- and K+ are the negative and positive controls. **M** – stands for a GenRuler 100bpPlus, used as a molecular marker (Galbács et al., 2024).

3.4. Testing of infections from individual plant samples with the five identified viruses of the study

To test how many individuals are infected with a particular virus in the pools, they were tested using RT-PCR, and presented according to the viruses.

Table 3. Summary of the sRNA HTS and RT-PCR diagnostics. The infection rate of the plant species is also indicated (Galbács et al., 2024).

Library	test	WSMV	BYSMV	BVG	ApGlV1	LDV1
1_M_US	sRNA HTS	-	-	-	-	-
	RT-PCR	3:11	-	3:11	-	-
2_ECG_US	sRNA HTS	-	-	-	-	-
	RT-PCR	5:8	-	-	-	-
3_SVCD_US	sRNA HTS	-	-	-	-	-
	RT-PCR	5:11	3:11	-	-	4:11
4_SH_U	sRNA HTS	-	-	-	-	-
	RT-PCR	-	-	-	3:5	-
5_M_BA	sRNA HTS	+	+	-	-	-
	RT-PCR	4:10	3:10	7:10	-	9:10
6_ECGSV_BA	sRNA HTS	-	-	+	-	-
	RT-PCR	6:6	-	1:6	2:6	3:6
7_Ma_BA	sRNA HTS	-	-	-	+	+
	RT-PCR	-	-	-	3:5	4:5

3.4.1. Phylogenetic analysis of WSMV

The sequence of the variant present in millet from US, which was also found in *E. crus-galli* of both locations in US and BA, *C. dactylon* in US and *S. viridis* in BA, and the variant present in millet at BA was 98.8% identical. They were also very similar and shared higher than 97% similarity with the variants sequenced in millet in 2019 (Pasztor et al., 2020). The variants sequenced during this study clustered into Clade B together with the previously sequenced European variants (Figure 7).

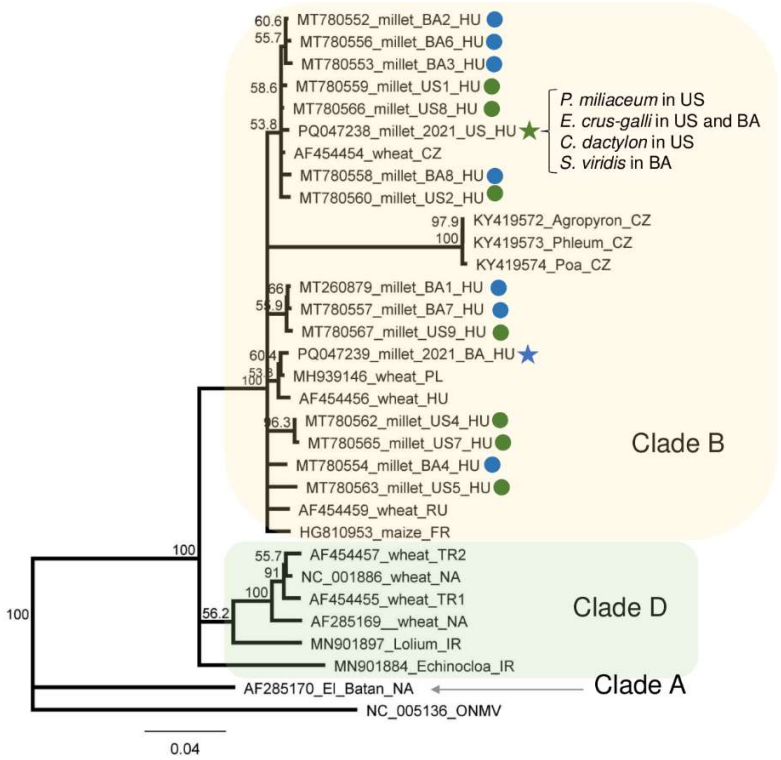


Figure 7. Phylogenetical analysis of the WSMV strains originating from BA and US. The phylogenetic tree was constructed based on the 755 nt long amplified and Sanger-sequenced, polyprotein-encoding (containing CP coding) part of the viral genome using the Neighbour-Joining analysis and the Jukes–Cantor model, with 1000x bootstrap replications. Bars represent 4% nucleotide diversity. Sequences originating from Pasztor et al. (2020) study are marked with circles, whilst sequences from this study are marked with stars. Green represents US, while blue represents BA. Sequences of the different strains are marked with their GenBank accession numbers, host species and countries of origin. HU—Hungary, CZ—Czechia; PL—Poland; RU—Russia; FR—France; TR—Turkey; NA—North America; IR—Iran. Green—US, blue—BA. ONMV—Oat necrotic mottle virus was used as an outgroup to root the tree (Galbács et al., 2024).

3.4.2. Phylogenetic analysis of BYSMV

The variant of BYSMV of the study was very similar and shared 99.78% similarity when clustered together with one of the variants found in 2019 (Pasztor et al., 2020) at the same place (Figure 8).

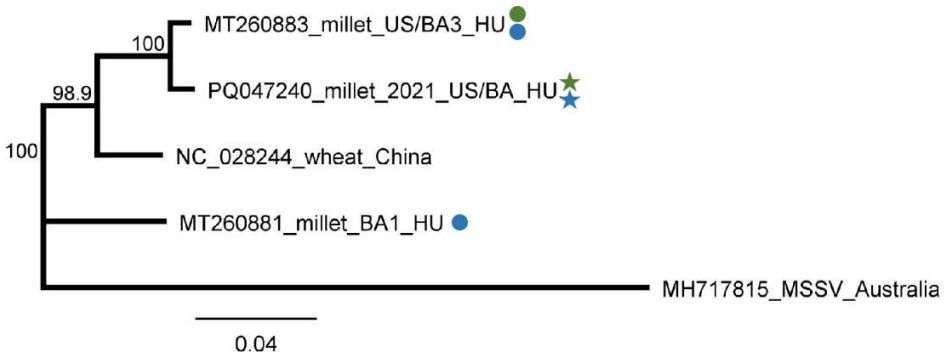


Figure 8**.** Phylogenetic analysis of the BYSMV strains originating from BA and US. The phylogenetic tree was constructed based on the 1287 nt long amplified and Sanger-sequenced, CP-coding part of the viral genome using Neighbour-Joining analysis and the Jukes–Cantor model, with 1000x bootstrap replications. Bars represent 4% nucleotide diversity. Sequences originating from Pasztor et al. (2020) study are marked with circles, whilst sequences from this study are marked with stars. Green represents US, while blue represents BA. Sequences of the different strains are marked with their GenBank accession numbers, host species and countries of origin. HU—Hungary. MSSV—maize sterile stunt virus (MSSV) was used as an outgroup to root the tree (Galbács et al., 2024).

3.4.3. Phylogenetic analysis of BVG

A nearly complete genome (5362 nt long) of the BVG strain in the *E. crus-galli* was amplified and sequenced. BVG variant sequenced on *E. crus-galli* is highly similar to the strains available in the GenBank, sequenced in different hosts at different locations (Figure 9a). It demonstrates the highest identity to a BVG variant sequenced in Great Britain from maize (98.7%) and in France from barley (98.3%). Sequence analysis of the smaller, 607 nt long part of the genome showed a similar, slightly unique trend (Figure 9b). The variants sequenced from millet in US, BA and *E. crus-galli* in BA were highly identical (higher than 99% identity) and were very analogous to the variant sequenced in 2019 (Pasztor et al., 2020). BVG variants were very similar, the variants sequenced in Europe showed higher than 97% identity, proposing a very conservative genome of the virus. The BVG variant from *E. crus-galli* clustered with the variant present in Great Britain, Slovenia and France. Although they were similar, the variants sequenced in 2019 (Pasztor et al., 2020) and in this study clustered distantly.

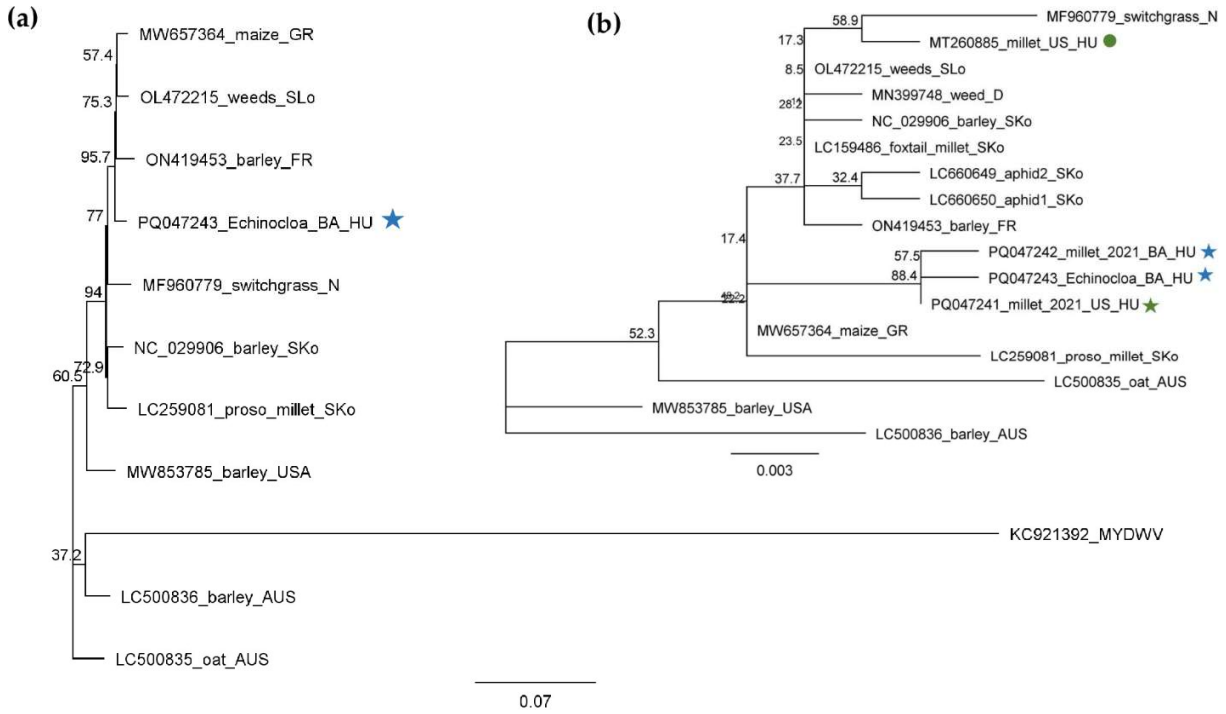


Figure 9. Phylogenetic analysis of the BVG strains originating from BA and US. The phylogenetic tree was constructed based on (a) nearly full (5362 nt long) BVG genomes and (b) the 607 bp long amplified and Sanger-sequenced, CP-coding part of the viral genome using the Neighbour-Joining analysis and the Jukes–Cantor model, with 1000x bootstrap replications. Bars represent (a) 7% and (b) 0.3% nucleotide diversity. Sequences originating from Pasztor et al. (2020) study are marked with circles, whilst sequences from this study are marked with stars. Green represents US, while blue represents BA. Sequences of the different strains are marked with their GenBank accession numbers, host species and countries of origin. GR—Great Britain; SLo—Slovenia; FR—France; HU—Hungary; N—the Netherlands; SKo—South Korea; AUS—Australia; D—Germany. MYDWV—Maize yellow dwarf virus was used as an outgroup to root the tree (Galbács et al., 2024).

3.4.4. Phylogenetic analysis of ApG1V1

SRNA HTS exclusively detected ApGlV1 infection in maize, as the size distribution of the small RNA reads obtained from ApGlV1 revealed that they were primarily at length of 20nt. The reads in 4_SH_U had peaks at 21 and 24nt, whereas in 6_ECGSV_BA, they were 24nt long. The sequences of the cloned products showed no distinction between the strains present in the three different species. The Hungarian variant was very similar to other sequences, the sequence identity of all available strains was higher than 98.2%. Beside their high similarity, the variants clustered together according to their geographical origin, regardless of their plant or insect host, suggesting

their on-site origin (Figure 10). The Hungarian variant however, clustered distantly with the sequence of ApGlV1 closest homologue: Tetranychus truncatus picorna-like virus 2 (TTPV2), which was employed as an outgroup. It is noteworthy that considering the amplified part, this virus is 92% highly identical to the ApGlV1 variants, and could be only a divergent strain of the same virus. This could also suggest that phylogenetically, the Hungarian variant is more connected to TTPlV2 than it is to the other variants, despite the fact that it is only 92% identical to the strain.

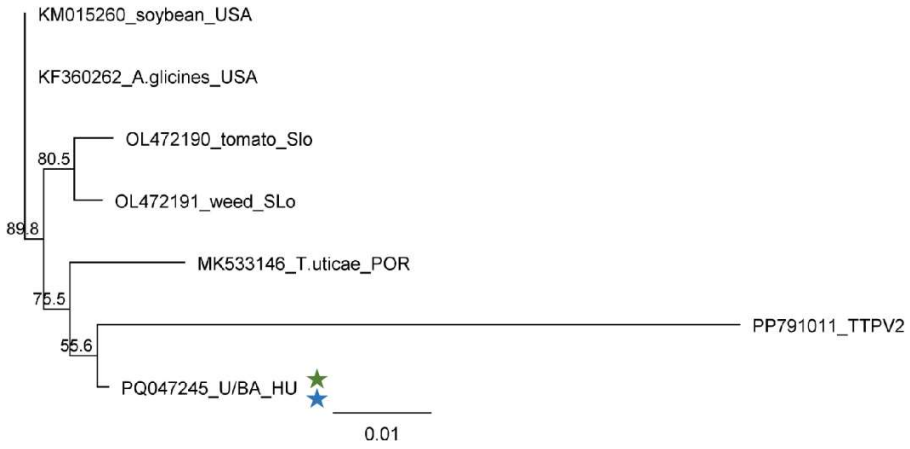


Figure 10**.** Phylogenetic analysis of the ApGlV1 strains originating from U and BA. The phylogenetic tree was constructed based on the 906 nt long amplified and Sanger-sequenced, VP4/VP3-coding part of the viral genome using the Neighbour-Joining analysis and the Jukes–Cantor model, with 1000x bootstrap replications. Bars represent 1% nucleotide diversity. Sequences originating from this study are indicated with stars. Green represents US, while blue represents BA. Sequences of the different strains are marked with GenBank accession numbers, host species and countries of origin. SLo—Slovenia; POR—Portugal; HU—Hungary. PP791011—Tetranychus truncatus picorna-like virus 2 (TTPV2) was used as an outgroup to root the tree (Galbács et al., 2024).

3.4.5. Phylogenetic analysis of LDV1

The outcome from the cloned and sequenced LDV1 obtained amplified products found that they are identical in the case of *S. viridis* and *P. miliaceum*, but slightly distinct from the other two species. The Hungarian variants were 97.9-99.4% identical to each other whilst the Slovenian variant 95.9-97.8% identical. The variants found in BA in *E. crus-galli* and maize clustered together, proving their common origin (Figure 11).

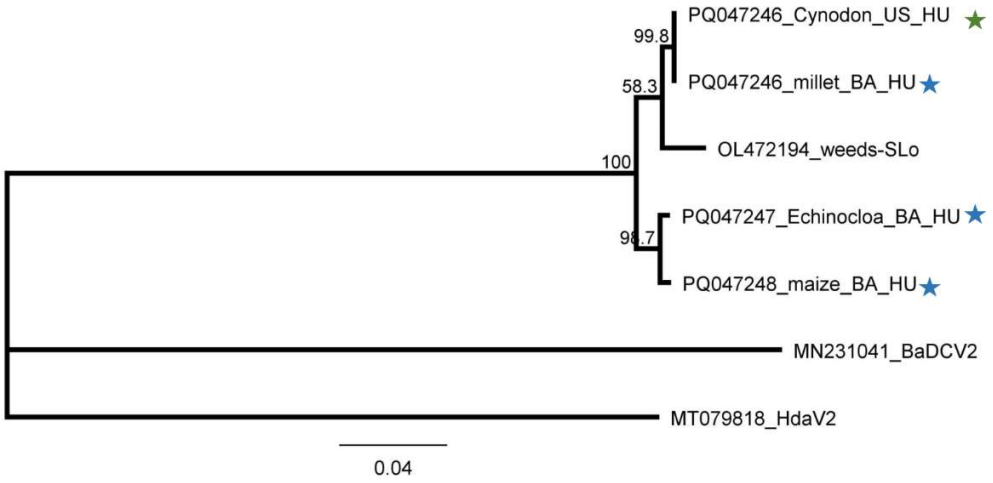


Figure 11. Figure 20**.** Virus diagnostics using RT-PCR to test the presence of LDV1 in the sampled individuals amplifying an 838 bp long part of the LDV1 genome using LDV1_5664F and LDV1_6480R primers. M stands for a GenRuler 100 bpPlus, used as a molecular marker. K− and K+ are the negative and positive controls. Red indicates positive individuals. The infection rate of the plant species is also indicated (Galbács et al., 2024).

3.4.6. Possible Viral Persistence of WSMV, BYSMV and BVG in two of the study locations

In a previous project of Pasztor et al. (2020) surveying two millet (*P. milliaceum*) populations, the study revealed the presence of WSMV, BYSMV and BVG in millet at US and BA. In this study, to investigate if these viruses could still be present and analyze its possible viral persistence (Table 4), the findings showed that in US and BA, WSMV was detected again in samples *P. milliaceum* (3:11) for US and (4:10) for BA, however, in a lower infection rate as compared to the study of Pasztor et al., 2020 who recorded higher rates and widespread of WSMV (7:10) in US and (9:10) infected *P. milliaceum* plants in BA. In US, *E. crus-galli* plants of (5:8) were WSMV infected while all *E. crus-galli* plants in the pool at BA were also infected. *C. dactylon* weeds of (5:11) were WSMV infected at US, and all samples of *S. viridis* in the pool at BA were significantly infected. In the case of BYSMV, the study revealed again the presence of this virus in *P. milliaceum* plants in BA (3:10). In US, the study revealed that *C. dactylon* weeds samples were also infected by BYSMV (3:11), indicating a possible new host of BYSMV. The study recorded an interesting outcome of BVG infecting *E. crus-galli* in BA (1:6).

Table 4. Virus persistence of WSMV, BYSMV and BVG in study locations of US and BA

Pasztor et al., 2020 US	***Panicum milliaceum* BA**	**In this study US**	***Panicum milliaceum* BA**	**New hosts identified in this study**
WSMV (7:10)	9:10	3:11	4:10	*Echinochloa crus-galli* (in both US and BA) *Cynodon dactylon* at US *Setaria viridis* detected at BA
BYSMV (3:10)	1:10	0:11	3:10	*Cynodon dactylon* weed of US
BVG (0:10)	1:10			*Echinochloa crus-galli* infected at BA

Plant virus persistence is a significance occurrence which needs to analyzed and addressed on crop fields. In its assessment whether in low or high infection rates, this activity could serve as the initiator and leads to the build up of plant viral populations in our crop fields. In the case of this study where these viruses have been detected in the same study location year on after its description, coupled with identifying new hosts of these viruses, the study has helped to reveal this occurrence and the information provided could be employed when planning and organizing an all-inclusive sustainable management practices against these viruses on the experimental fields.

4. NEW SCIENTIC RESULTS

1. We confirmed that WSMV infects *Cynodon dactylon* and *Setaria viridis* and provided partial sequence of the infecting WSMV strain.
2. We first described *Cynodon dactylon* as a new host of BYSMV and *Echinochloa crus-galli* as a new identified host of BVG.
3. We first described the presence of ApGlV1 in Hungary. *Sorghum halepense*, *Echinochloa crus-galli* and maize have been identified as new potential hosts of the virus.
4. We detected LDV1 first time in Hungary, and identified *Setaria viridis*, *Echinochloa crus-galli*, *Panicum milliaceum* and *Zea mays* as its new potential hosts.

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