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FACULTY OF HORTICULTURAL SCIENCE
BUDAPEST

Molecular Detection of Peach-associated Luteovirus (PaLV) in Peach Trees

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1. USED ABBREVIATIONS

HTS: High-throughput sequencing

NGS: Next generation sequencing

IWI: Indexing on woody indicators

PCR: Polymerase chain reaction

RT-PCR: Reverse transcription polymerase chain reaction

ELISA: Enzyme-linked immunosorbent assay

TBIA: Tissue blot immunoassay

QCMI: Quartz crystal microbalance immunosensors

DAS: Double antibody sandwich

DAC: Direct antigen-coating

TAS: Triple antibody sandwich

PAS: Protein A-sandwich

PaLV: Peach-associated Luteovirus

NSPaV: Nectarine stem pitting-associated virus

PPV: Plum pox virus

PNRSV: Prunus necrotic ringspot virus

CMV: Cucumber mosaic virus

CTV: Citrus tristeza virus

PLRV: Potato leaf roll virus

PVX: Potato virus X

PVY: Potato virus Y

DNA: Deoxyribonucleic acid

cDNA: complementary DNA

dNTPs: Deoxynucleotide

RNA: Ribonucleic acid

dsRNAs: Double-stranded RNA

rRNA: ribosomal ribonucleic acid

sRNA: small RNA

RNAseq: RNAs sequencing

ORFs: Open reading frames

CTAB: Cetyltrimethylammonium bromide

EDTA: Ethylenediaminetetraacetic acid

SDS: Sodium dodecyl sulfate

SSTE: Sodium dodecyl sulfate–Tris-HCl–EDTA

TAE: Tris base, acetic acid and EDTA

TBE: Tris base, boric acid and EDTA

Bp: Base pair

MQ: Milli-Q

NCBI: The National Center for Biotechnology Information

BLASTn: Nucleotide Basic Local Alignment Search Tool

FASTA: A text-based format for representing either nucleotide sequences or amino acid sequences

MEGA: Molecular Evolutionary Genetics Analysis

LB: Luria-Bertani

UV: Ultraviolet

2. INTRODUCTION

Peach cultivars are clones, propagated by grafting to guarantee true-to-type trees (LaRue, 1989). Therefore, when using infected plant material, peach foundation orchards are definitely exposed to a wide spectrum of 20 different viruses and virus-like agents that have been identified in peach to date (Jo *et al.*, 2018).

Over the last years, the use of high-throughput sequencing (HTS) have significantly facilitated the task of viruses' detection (Maliogka *et al.*, 2018). In 2017, *Peach-associated Luteovirus* (PaLV) has first been identified by an HTS analysis in two peach accessions from the Republic of Georgia and Spain, and has been considered a member of a new species of the genus *Luteovirus* (Wu *et al.*, 2017). Subsequently, the presence of PaLV was reported in both of Italy (Sorrentino *et al.*, 2018) and China (Zhou *et al.*, 2018).

In Hungary, fruit trees nurseries have adopted the so-called indexing on woody indicators (IWI) method to ensure a virus-free planting material before being distributed to its last destination in farmers' fields. However, IWI has drawbacks in terms of the time needed to obtain the results (1 up to several years in the field conditions) (Gilles and Bormans, 1985), and more importantly the method bears a questionable sensitivity towards the viruses of interest.

In this research, I have used molecular biology means, reverse transcription polymerase chain reaction (RT-PCR) technique namely, in order to assess the IWI efficiency by comparing its results with those of the molecular detection of PaLV.

3. LITERATURE REVIEW

3.1 Peach (*Prunus persica)*

Peach (*Prunus persica*) is a deciduous tree native to the region of Northwest China where it has been first domesticated and cultivated (Faust, Timon and others, 1995). Currently, there are approximately 1.5 million hectares of peaches and nectarines in production worldwide, 51% of which only in China (FAOSTAT, 2017). Furthermore, peach is ranked third of most produced temperate tree fruit species following apple and pear (Byrne et al., 2012), with an estimated annual production of approximately 20 million tons (Faostat, 2016).

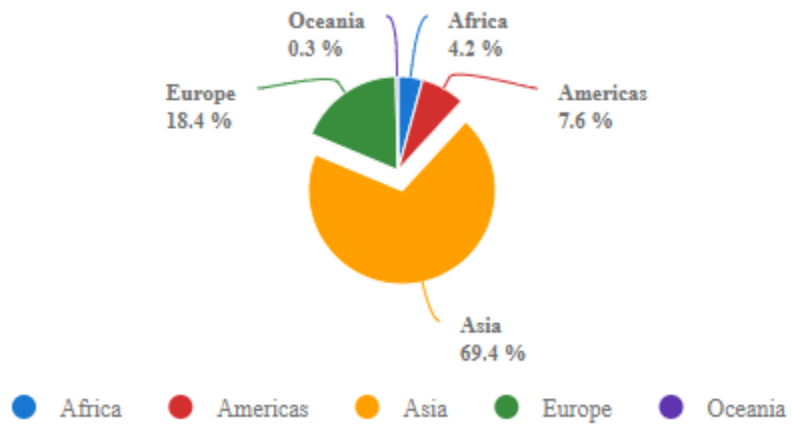

Figure 1. Production share of Peaches and nectarines by region (FAOSTAT, 2017)

As a vegetatively propagated plant, grafting is used to maintain fruit traits. This exposes peaches and nectarines to a wide spectrum of more than 20 different viruses and viroids that have been identified to date (Jo et al., 2018), some of them are considered as regulated viruses in Hungary such as *Plum pox virus* (PPV; genus *Potyvirus*) and *Prunus necrotic ringspot virus* (PNRSV; genus *Ilarvirus*) (Baráth et al., 2018).

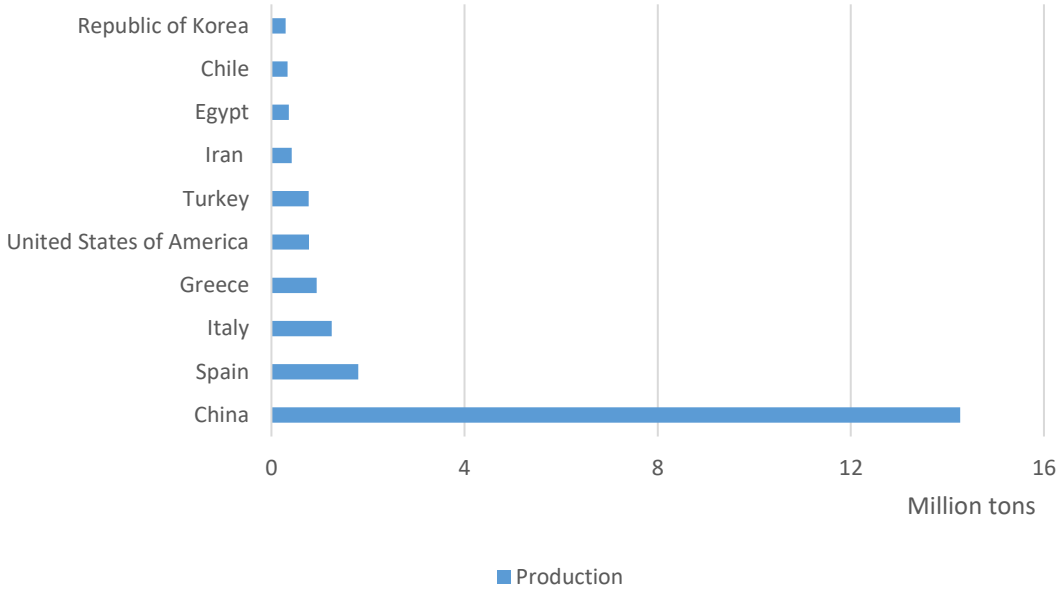

Figure 2. Production of Peaches and nectarines: top 10 producers (FAOSTAT, 2017)

Costs of damage caused by viral diseases range from nearly nothing to unmarketable fruits or death of plantations (Fridlund and others, 1980). Many fruit tree viruses have an elongated effect on both the growth and physiology of their hosts where the economic consequences might only be realized years after the infection.

In Nectarine (*Prunus persica cv. nectarina*), for example, some viruses are associated with symptoms such as *Plum pox virus* and *Peach mosaic virus*, whilst viruses such as *Cherry virus A* are not linked to acute symptoms (Villamor et al., 2016).

3.2 Peach major viruses

Table 1. Major viruses affecting peach fruit trees (Barba, Ilardi and Pasquini, 2015)

Family	Genus	Species	Transmission
	Trichovirus	*Apple chlorotic leaf spot virus*	Mainly by grafting
Betaflexiviridae		*Cherry mottle leaf virus*	Grafting *Eriophyes inaequalis*
	unassigned	*Cherry green ring mottle virus*	has no known vector and is not seed-transmitted
		Prunus necrotic ringspot virus	Vegetative propagation Seed-transmitted Pollen-transmitted
Bromoviridae	***Ilarvirus***	*Apple mosaic virus*	Vegetative propagation
		Prune dwarf virus	Grafting Pollen-transmitted Seed-transmitted
Closteroviridae	***Ampelovirus***	*Little cherry virus 1*	Grafting
		Little cherry virus 2	Grafting *Phenacoccus aceris* *Pseudococcus maritimus*
Potyviridae	***Potyvirus***	*Plum pox virus*	Vegetative propagation *Aphis spiraecola* *Myzus persicae*

Furthermore, *peach virus D* (a putative new member of the genus *Marafivirus*) and *peach virus T* (a new member of the genus *Marafivirus* in the family *Tymoviridae*) were recently identified in peach using Next Generation Sequencing (NGS) (Igori, Lim, et al., 2017) (Jo et al., 2018).

The vegetative plant material of stone fruit trees, such as peaches, are globally exchanged for commercial purposes carrying a potential risk of mobilizing pests and diseases into other geographic regions. Most countries, therefore, have adopted their own measures and protocols of post-entry quarantine utilizing serological or molecular diagnostic assays that screen only for known pathogens prevalent in the country of origin or elsewhere, and bioassays on indicators (Bag et al., 2015).

3.3 Virus diagnostic methods

Conventional virus detection methods such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), nucleic acid hybridization or microarray are not highly efficient in detecting novel viruses or virus variants since they require prior knowledge of the potential pathogens (Zheng et al., 2017), however, they provide rapid diagnoses for known viruses and viroids (Wu et al., 2015).

3.3.1 For known viruses

3.3.1.1 Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is the most used serological detection methods, but others such as tissue blot immunoassay (TBIA) and quartz crystal microbalance immunosensors (QCMI) were also developed, which use virus-specific antibody developed in animals in response to antigen (Jeong, Ju and Noh, 2014).

The term ELISA was first coined in 1971 by Engvall and Perlmann who described these immunoassays utilizing enzyme labels. However, it was until 1976 when ELISA has been first applied in plant virology (Clark, Lister and Bar-Joseph, 1986).

ELISAs have been broadly used in the last three decades yet their use has decreased due to some drawbacks in terms of antibodies' availability, their production costs, and the need for samples of large volume to capture the antigen of interest (Jeong, Ju and Noh, 2014).

Efforts were made for ELISA development, where we can distinguish two wide types of ELISA procedures, the direct ELISA and the indirect ELISA, having the same theoretical background; however, they differ in how to detect the antigen-antibody complex (Naidu and Hughes, 2003).

Whilst Direct ELISA, also known as double antibody sandwich (DAS), is considered as highly strain-specific, the indirect methods, such as Direct antigen-coating (DAC), Triple antibody sandwich (TAS) and Protein A-sandwich (PAS) can be used to detect a broad range of viruses, therefore, they are preferred in surveys and quarantine measures for economic and practical reasons (Naidu and Hughes, 2003).

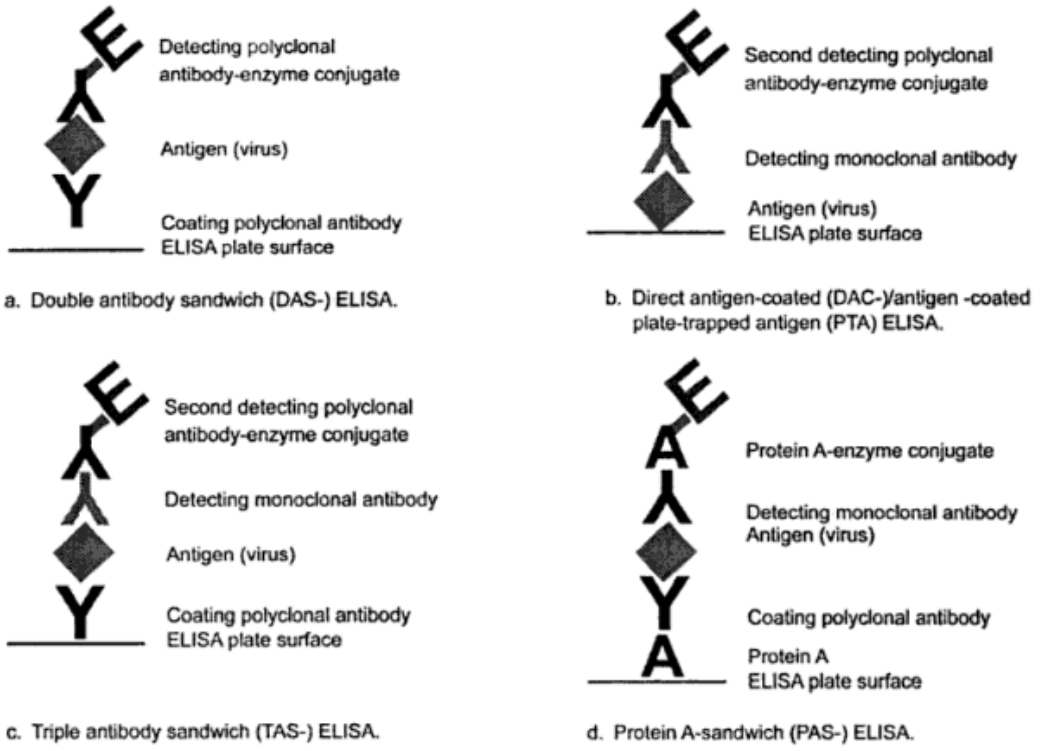

Figure 2. DAS-, DAC-, TAS-, and PAS-ELISA are four types of ELISA commonly used for plant virus detection (Naidu and Hughes, 2003)

ELISAs are usually done in microtiter plates made of either inflexible polystyrene or flexible polyvinyle chloride binding antibodies or proteins with enzyme-substrate reaction, therefore, optimizations are needed to get accurate and reproducible results. When it comes to estimate the infection level in ELISA reaction, the degree of coloration (the optical density) is to be determined (Naidu and Hughes, 2003; Jeong, Ju and Noh, 2014)

Sensitivity, usually with detection range of 1-10 ng/ml, the ability to handle hundreds of samples at once, and the potential for semi-automation (Clark, Lister and Bar-Joseph, 1986; Jeong, Ju and Noh, 2014) are among many advantages that ELISAs offer. Indeed, ELISA has been utilized to detect numerous viruses such as *Cucumber mosaic virus* (CMV), *Citrus tristeza virus* (CTV), *Potato leaf roll virus* (PLRV), *Potato virus X* (PVX), *Potato virus Y* (PVY)(Jeong, Ju and Noh, 2014) and PPV.

ELISA's specificity originates from specific antibody that has been raised against a virus coat protein (Clark, Lister and Bar-Joseph, 1986). Although many additives have been added to the extraction buffer to increase ELISA's specificity, ELISA is unable to properly differentiate very close viral strains (Jeong, Ju and Noh, 2014).

3.3.1.2 Detection by Polymerase Chain Reaction methods (PCR)

The use of PCR became common in plant pathology discipline with the introduction of *Thermus aquaticus* (Taq) DNA polymerase in 1988. The relative stability of this enzyme at DNA-melting temperatures has eliminated the need for enzyme refill after each cycle of synthesis reducing PCR costs and allowing an automated thermal cycling. The method offers both narrow and broad spectrums of selectiveness, according to the choice of primers, it facilitates the detection of a single pathogen or many members of a group of related pathogens, unlike serology, at a lower cost (Henson and French, 1993).

These methods first have been introduced in the early 1990s (VUNSH, Rosner and Stein, 1990), and were the most successfully exploited for virus detection. Especially when using gel-electrophoresis for resolution of the results (conventional PCR-based assay), the specificity can be often achieved whilst the sensitivity is frequently below expectation.

Factors such as the optimization of the reaction mixture composition and temperature cycling regime affect the specificity and efficiency of DNA amplification by PCR. Therefore, designing a set of primers followed by adjusting the primer and buffer salt concentrations, thermal cycle times and temperature are needed to reach the desired sensitivity and selectivity (Henson and French, 1993).

Due to a range of practical issues, mainly problems with post-PCR contamination, very few conventional PCR methods have been deployed routinely in diagnostic laboratories, as small quantities of DNA released into the laboratory environment upon opening the tubes after thermal cycling, which could be detected by the PCR method resulting in false positive results. Real-time PCR, a closed-tube PCR assay, has solved that problem and quickly been adopted for diagnostic applications where the fluorescent signal is generated within the closed PCR tube and detected either during amplification (real-time) or at the end of it without opening the tube eliminating therefore the risk of contamination (Boonham et al., 2014).

3.3.2 For unknown viruses

Both indexing on woody indicators (IWI) and NGS analysis are used for this purpose. In a study compared NGS with IWI for optimal detection of Grapevine viruses, NGS has been found to be superior in terms of sensitivity to IWI and more reliable since the latter recorded a detection failure in 8.3% of the overall indicator tests. Whilst NGS took only weeks, IWI required 2 years to obtain results in California warm climate , it would take much more time in cooler areas, thus a significant savings of time and costs would be achieved with NGS analysis (Al Rwahnih et al., 2015).

3.3.2.1 Indexing on woody indicators.

Field Indexing on woody indicators is expensive and labor-intensive; it requires large, field-grown indicator trees for bud inoculations and a period of 2 years to obtain results. Herbaceous indicators, on the other hand, are less accurate and not easily handled in comparison with the woody indicators, except from Chenopodium quinoa that is able to detect a few extremely mild strains of the apple chlorotic leaf spot and apple stem grooving viruses. For faster results, less than 4 weeks, indexing is performed inside a greenhouse space for 20 indicators (Fridlund and others, 1980).

Double budding is the main indexing method, where two inoculum buds are budded low on a healthy rootstock, whist a bud of a specific indicator is budded directly above the two inoculum buds. Therefore, any infecting virus is graft-transmitted from the inoculum buds to the healthy rootstock and subsequently to the indicator. In order to force the indicator bud to grow, the healthy rootstock is cut back, and lastly the resulting foliage of the indicator is checked for characteristic symptoms (Fridlund and others, 1980).

3.3.2.1.1 Greenhouse indexing

In the greenhouse conditions, constant temperatures are maintained where the healthy rootstocks, about 6-7 mm in diameter, are planted in plastic pots (20 index trees can be grown per 1 m²) with a normal soil mix and some nitrogen fertilizer added later (Fridlund, 1979).

The budding can be done when the seedlings are just beginning to break, then inoculations are made by simultaneously double budding two inoculum and one indicator bud to each healthy seedling. The seedlings are cut back, one week after budding, to force indicator buds to grow (Fridlund, 1979).

, The indicators are usually observed for symptoms four weeks after inoculation, which is equivalent to maintaining an indicator in the field for one year. However, when a field equivalent of two or more years is required, the indicator shoot is cut back after four weeks to about 7-8 cm and completely defoliated. Within an additional six weeks, refoliation will happen and all virus symptoms normally requiring two or more years in the field to develop will be expressed (Fridlund, 1979).

3.3.2.1.2 Field indexing of stone fruits (*Prunus*)

To avoid loss of the candidate clone in case of mechanical accident or natural infection, three trees of the candidate clone are propagated by budding on healthy seedlings simultaneously with the preliminary indexing in order to discard any virus-infected candidate from the beginning of the procedure (Fridlund and others, 1980).

These trees are kept until reaching suitable size to provide sufficient budwood for indexing on the standard indicators. Once the three trees of the candidate has reached that size, one of them is selected for indexing on 8 indicators of stone fruit viruses. In case the selected tree appeared to be virus-free, it is designated the nucleus mother tree of the candidate clone and maintained permanently, whereas the other unindexed trees are then discarded (Fridlund and others, 1980).

From the nucleus mother tree, two daughter trees are propagated and planted in isolated field repositories. The daughter trees are then used to produce the budwood that is distributed. The nucleus mother trees and the daughter repository trees are reindexed occasionally to detect any probable natural infections (Fridlund and others, 1980).

3.3.2.2 High-throughput (Next generation) sequencing

Although the reliability of this technique, also known as High-Throughput Sequencing (HTS), has been proven for virus discovery in many agricultural crops, some challenges are still facing the deployment of HTS as a detection tool where aspects of validation such as sensitivity, specificity, reproducibility and repeatability have to be taken into consideration (Maree et al., 2018) .

HTS gives a complete view of the viral phytosanitary status of a plant in a single assay, providing an insight on the virus population structure, ecology and evolution. Furthermore, the resulted data could be analyzed by multiple end-users or may be re-analyzed as databases are expanded (Maree et al., 2018).

In terms of sensitivity, it is directly linked to the proportion of viral RNAs among the cellular RNAs of the sample, the efficiency of the enrichment strategy, and the sequencing depth as well as the performance of the bioinformatics analysis. However, unlike other methods, where the specificity is assessed by testing the performance of reagents such as primers or antibodies, the specificity of HTS is assessed by verifying inclusivity and exclusivity of the database(s) of sequence used in the bioinformatic approach (Maree et al., 2018).

Moreover, selecting the library type of NGS should be in accordance with the study goal, for example, mRNA based library might be insufficient when targeting viruses without poly-A tail (Jo et al., 2018). However, small RNA (sRNA)-based virus diagnostics and Long viral RNAs sequencing (RNAseq), two different

applicable approaches based on next generation sequencing, are able to detect all plant viruses (Santala and Valkonen, 2018).

3.3.2.2.1 Small RNA (sRNA)-based virus diagnostics

This approach has proven to be highly efficient in plant virus detection. It exploits a fundamental antiviral defense mechanism called RNA interference, which is activated upon the viral infection by both RNA and DNA viruses of eukaryotes. During antiviral silencing, dsRNAs of viral origin are cleaved by DICER and DICER-like enzymes into small interfering RNAs with sizes of 21 to 24 nucleotides, which can be readily detected by deep sequencing of host small RNAs (Zheng et al., 2017).

Small interfering RNAs, generated by the host antiviral defense, could represent the entire genome of the infecting viruses or virus-like agents. Therefore, performing a deep sequencing followed by bioinformatics analysis of the small RNA population allow us to reconstruct the complete virome even during mixed viral infections (Pooggin, 2018).

In an investigation to compare the sensitivity of sRNA-based method with the established real-time PCR concerning PVY and PVA, the results showed a 10-fold higher amount of viral RNA is needed to detect these viruses when using sRNA-based method in a *de novo* manner. However, when test is done for known viruses, sRNA-based method showed 10 times more sensitivity than the real-time PCR-based method (Santala and Valkonen, 2018).

3.3.2.2.2 RNA sequencing (RNA-seq)

Using this method, viruses are identified by directly sequencing total RNA from the host plants (Wu et al., 2015). Whilst small RNAs can be directly sequenced after a step of adaptor ligation, in RNA-seq the larger RNA molecules have to be subjected to a fragmentation step into 200-500bp, which could be done either on RNA level or on cDNA level by RNA hydrolysis or DNase 1 treatment respectively (Wang, Gerstein and Snyder, 2009).

However, different strategies have been developed to enrich the deep-sequencing reads specific to viruses and/or viroids, such as the rRNA depletion from total RNA preparations, and similarly by sequencing only the polyadenylated transcripts that have led to discover seven new RNA viruses and two DNA viruses (Wu et al., 2015).

Another strategy based on sequencing the double-stranded RNAs, synthesized by RNA viruses and viroids as replicative intermediates, has dramatically increased the amount of reads specific to viruses and

viroids (Wu et al., 2015), and reduces RNA of host origin (Bag et al., 2015). Six new RNA viruses and one DNA virus have been discovered following this method (Wu et al., 2015) including two proposed *capilloviruses*, a *potivirus* species, and a *partitivirus* species that have been found in 4 species of Donkey Orchids (Wylie et al., 2013). Furthermore, compared with total RNA sequencing, a study found that virus reads increased from 2% to 53% (Wu et al., 2015).

RNAs sequencing showed superiority over the sRNA-based method for most the tested linear RNA viruses (except from PVY), especially when the sRNA-based approach failed in detecting a putative novel *Cytorhabdovirus*. However, sRNA-based approach achieved higher yields of viral sequences for viroids and viruses of circular ssDNA where a better performance has been reported (Pecman et al., 2017).

3.4 Peach luteoviruses

The members of the genus Luteovirus are of economic importance, characterized by 25-30 nm isometric particles with a single strand of plus-sense RNA from 5.3 to 5.9 kb, and are transmitted by aphids in a circulative, non-persistent manner (Wu et al., 2017). "Lutioviruses usually contain five major Open Reading Frames (ORFs): ORF1 and ORF2 encode an RNA-polymerase (RdRP) via ribosomal -1 frameshift, ORF3 encodes a capsid protein, ORF4 a movement protein, while ORF5 is translated into an aphid transmission factor by read-through of the stop codon in another ORF. All luteoviruses possess a small ORF3a protein responsible for long-distance movement, and one or two small and variable ORFs at the 3' end of the genome" (Lenz et al., 2018).

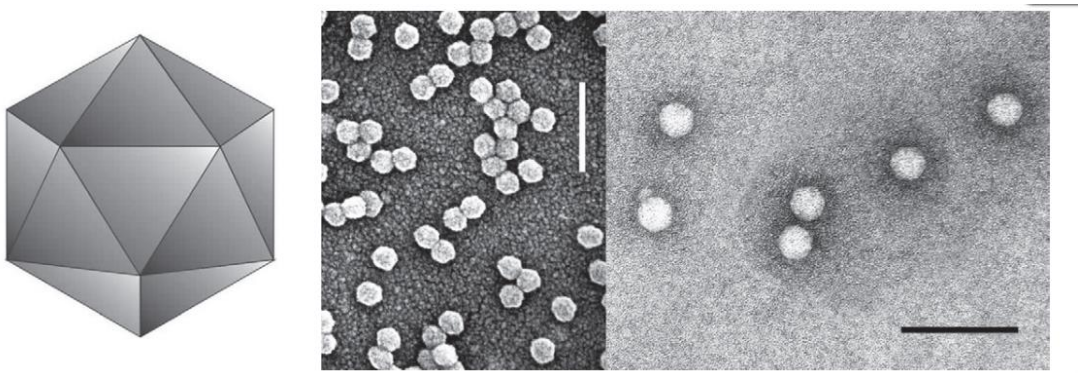

Figure 3. (Left) Diagram of the proposed structure of luteovirus particles. (Center) Negative contrast electron micrograph of particles of barley yellow dwarf virus-PAV and (right) pea enation mosaic virus-1. Bars represent 100nm (King et al., 2011).

3.4.1 Peach-associated Luteovirus

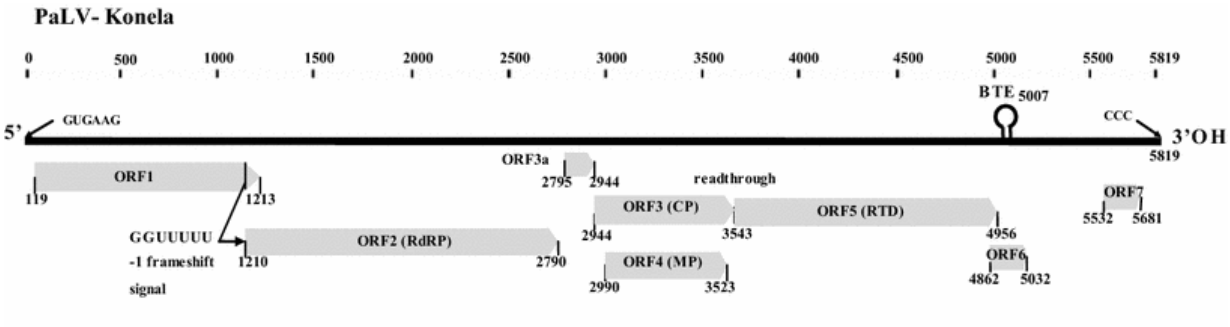

Figure 4. Genomic organization of *peach-associated luteovirus* (PaLV- Konela) (Wu et al., 2017).

Identifying PaLV was the result of high-throughput sequencing analysis in two peach accessions from the Republic of Georgia and Spain, where the complete sequences of both PaLV isolates were determined revealing a genomic structure of eight open reading frames, with 58-74% identity to those of other members of the genus *Luteovirus* (Wu et al., 2017).

In respect to PaLV incidence, the virus might be a common one since it has been detected, using RT-PCR, in 14 out of 36 accessions from the peach germplasm collection at the USDA-Appalachian Fruit Research Station (Wu et al., 2017).

In terms of symptomatology: Both the PaLV-infected peach accessions (Konela and IVIM18) were asymptomatic, however, mild mosaic symptoms have developed on young leafs of a graft-inoculated GF 305 peach indicator (Wu et al., 2017).

3.4.2 Nectarine stem pitting-associated virus (NSPaV)

NSPaV was first described in the United States of America, and recently detected in each of China, Japan, South Korea and Hungary (Igori, Baek, et al., 2017).

The virus was discovered in California by performing an NGS analysis, based on the double-stranded RNA extracts, in nectarine trees propagated from budwood of French origin. Although the imported budwood exhibited stem-pitting symptoms, they have been cleared through the Californian quarantine procedures, what therefore stresses the significance of NGS adoption as a key tool to evaluate the plant health status in the current post-entry quarantine measures (Bag et al., 2015).

By using RT-PCR with reported primers that were designed based on an available partial coat protein sequence in Genebank, the virus was detected in 54 out of 54 samples of both symptomatic and asymptomatic nectarine and non-nectarine cultivars from 3 Chinese provinces (Lu et al., 2017).

In Hungary, NSPaV was reported by an NGS analysis in a peach sample showed severe yellow leaf symptoms, and confirmed by RT-PCR besides PPV, in 13 out of 13 additional samples from the same orchard (Krizbai et al., 2017).

4. MATERIALS AND METHODS

4.1 Biological material

Samples were collected at the Virology station at Velence (Hungary) in June 2018 from the biotest of 12 different *Prunus persica* varieties. 12 samples were collected from the shooted varieties (they were previously grafted on rootstocks). The other 12 samples were collected from their corresponding GF31 woody indicators. Sampled shoots were either asymptomatic or showed some yellowing but never virus specific symptoms. Sampled leaves were collected separately from each shoot and were stored at -80°C until used.

Table 2. Description of the samples.

Sample number	Peach Varieties	Sample number	Woody Indicators
1	Flavortop	13	1/GF31
2	Nektar H	14	2/GF31
3	Venus	15	3/GF31
4	Incrocio Pieri	16	4/GF31
5	Elberta	17	5/GF31
6	Cresthaven	18	6/GF31
7	Redhaven	19	7/GF31
8	Early redhaven	20	8/GF31
9	Champion	21	9/GF31
10	Suncrest	22	10/GF31
11	Apolka	23	11/GF31
12	Aranycsillag	24	12/GF31

4.2 RNA isolation

Total RNAs were extracted from 150-200 mg of each frozen leaf sample using a CTAB-based protocol (Gambino, Perrone and Gribaudo, 2008) . Purified RNAs were dissolved in 25 µl of RNAse-free deionized water and kept frozen in -80°C until used.

4.2.1 CTAB-based RNA extraction protocol

For RNA extraction, Cetyltrimethylammonium bromide (CTAB)-based protocol of Gambino and colleagues was used (Gambino, Perrone and Gribaudo, 2008).

Pestles, mortars and all glassware used in the isolation of total RNA from plant material were kept overnight at 180°C; plasticware was autoclaved before use, whereas solutions used in RNA extractions were sterilized to inactivate RNases.

For the CTAB-based procedure, 850 µL of extraction buffer [2% CTAB, 2.5% PVP-40, 2 M NaCl, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA pH 8.0 and 17 µL of β-mercaptoethanol added just before use] were heated at 65°C in a 2ml microcentrifuge tube.

The sample (150-200 mg), powdered in liquid nitrogen, was added to the extraction buffer and the tube was incubated at 65°C for 10 min. An equal volume of 850 µL chloroform: isoamyl alcohol (24:1 v/v) was added and the tube was inverted vigorously and centrifuged at 10,000 rpm for 10 min at 4°C.

The supernatant was transferred to a new 2ml microcentrifuge tube and LiCl (3 M final concentration) was added. The mixture was incubated in ice for 30 min and RNA was selectively pelleted after centrifugation at 13,000 rpm for 20 min at 4°C. The pellet was resuspended in 450 µL of SSTE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS, 1 M NaCl) pre-heated at 65°C, an equal volume of chloroform:isoamyl alcohol was added and the mixture was centrifuged at 10,000 rpm for 10 min at 4°C.

The supernatant was transferred to a new 1.5 ml microcentrifuge tube and the RNA was precipitated with 280 µL isopropanol and 30 µL 4M sodium-acetate and incubated for 5 minutes at room temperature, and centrifuged at 13,000 rpm for 20 min at 4°C.

The pellet was washed with 1 ml ethanol (70%), centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was removed, and samples were dried at speed vac for 2 minutes.
Dried samples were resuspended in MilliQ pure water and kept on ice until used.

4.3 Examination of the quality of the extracted RNA

Purity and quality of the RNA were detected by 1.2% agarose gel electrophoresis in TBE buffer, stained with ethidium bromide and visualized under UV light.

RNA samples were prepared by mixing 2 µl of the extracted RNA with 3 µl FDE loading dye and 1 µl sterile water. Samples were denatured at 65°C for 5 min, and kept on ice until used. 6 µl denatured RNA sample were loaded into 1.2% agarose gel, and gel electrophoresis was applied. Quantification of the samples was determined using NanoDrop spectrophotometer.

4.3.1 Preparation of the agarose gel

The equipment and supplies necessary for conducting agarose gel electrophoresis include electrophoresis chamber, power supply, gel casting trays, wells comb, electrophoresis buffer TBE, loading die, ethidium bromide and trans illuminator (an ultraviolet light box) that was used to visualize ethidium bromide stained nucleic acids in gels.

A quantity of 3.6 g agarose was added to 300 ml of 1x TBE (Tris base, boric acid, EDTA), and heated in a microwave oven until completely melted. 0.7 µl (10 µg/µl) of ethidium bromide was added to 25 ml of agarose gel to facilitate the visualization of RNA after electrophoresis. After cooling down the gel was poured into a casting tray containing a wells comb and allowed to solidify at room temperature.

4.3.2 Running the agarose gel

After the gel has solidified, the gel was inserted into electrophoresis chamber and covered with 1xTBE buffer. The prepared RNA samples were then pipetted into the gel wells. Finally, samples were run at 110 V.

4.3.3 Visualization of RNA fragments

When the dye line was about at 75-80% of the way down the gel, the electrophoresis was completed, power supply turned off and the lid of the gel box was removed. Finally, for screening and observing the migration of the RNA fragments, “Bio-RAD chemidoc MP imaging system” was used.

4.4 cDNA synthesis

Thermo Scientific “Revert Aid™ First Strand cDNA Synthesis Kit” was used to synthesize first strand cDNA from total RNA template.

4.4.1 First strand cDNA synthesis

0.25 µl of Random Hexamer Primer, and 2.75 µl of total RNA (the maximum allowed quantity in the reaction, due to the lack of highly concentrated RNA in my samples) were added into a sterile and nuclease-free 0.5 ml microcentrifuge tube, on ice, and centrifuged briefly.

RNA was denatured by incubating the mixture at 65°C for 5 minutes followed by chilling on ice.

A reaction mixture was prepared by adding 1 µl 5x Reaction Buffer, 0.5 µl 10 mM dNTP, 0.25 µl RiboLock RNase Inhibitor, and 0.25 µl RevertAid Reverse Transcriptase. The reaction mixture was mixed gently and centrifuged briefly.

The reaction mixture was incubated as follows:

- 25°C for 10 minutes
- 42°C for 60 minutes
- 45°C for 10 minutes
- 70°C for 10 minutes for reaction termination

10x dilution of the synthesized cDNA was made: 2 µl of the synthesized cDNA was added to 18 µl of MQ water, and stored at -20°C until used.

4.5 Control PCR amplification

To test the quality of the generated cDNA, an endogenous internal control (β-actin housekeeping gene) was utilized to perform a control PCR, as follows:

- A reaction mixture was prepared by adding the following reagents in each tube: 9.4 µl MQ water, 3 µl 5X Phire Green Reaction Buffer, 0.75 µl β-actin gene Forward primer, 0.75 µl β-actin gene Reverse primer, 0.3 µl 10 mM dNTPs, 0.3 µl Phire Hot Start DNA Polymerase and 0.5 µl template 10x RT.
- PCR program was performed in a thermal cycler according to the following steps in the following table.

Table 3. Control PCR amplification program.

Step	Temperature °C	Time	Number of Cycle
Initial Denaturation	98	30 s	1
Denaturation	98	10 s	35
Annealing	55	10 s	35
Extension	72	20 s	35
Final extension	72	1 min	1
Hold	4	∞	-

4.6 Diagnostics of Peach-associated Luteovirus (PaLV)

4.6.1 Primer design

The Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was used in order to find PaLV-homogenous sequences by providing the database with the accession number of the reference genome of PaLV.

Each of the PaLV reference genome, the obtained PaLV-homogenous sequences, and the consensus sequence of the small RNA NGS data (generated by the NARIC Diagnostics Lab), altogether were aligned by using the Clustal Omega (multiple sequence alignment program). Subsequently, a list of reported PaLV primers were searched against the aligned sequences and only those matching conservative regions were kept and then modified to suit the consensus sequence.

Furthermore, the potential primers were tested by PCR Primer Stats online tool for parameters such as percent GC content, melting temperature, self-annealing and hairpin formation. Finally, the NCBI's BLAST was used to show whether the chosen primers are specific to the targeted sequence and do not bind any region of the peach genome.

Table 4. Newly designed PaLV-specific primers that were used for the RT-PCR diagnostics.

Virus	Primer Name	Primer Sequence (5'-3')	Position on the reference genome	Size of the amplified product	Function of the amplified region	Genome used as a reference	Reference*
PaLV	PaLV_F_2430	CACTGGTCAATGTGGCATGATCC	2430	1131bp	coat protein	NC_034970.1	this work
	PaLV_R_3561	CTGAGGAGCTGCGTCTACC	3561				(Wu *et al.*, 2017)

4.6.2 Gradient PCR

The thermal gradient feature, available on thermal cyclers, allows identification of the Best Annealing Temperature (BAT) for the primer set in a single run. Thus, in order to identify the BAT for the PaLV virus specific primers (PaLV_F_2430 and PaLV_R_3561), a gradient PCR was performed using a temperature gradient of 5 different annealing temperatures (ranged from 50°C to 65°C) to determine a single annealing temperature that will provide efficient, specific amplification of the targeted PaLV coat protein sequence.

The reaction mixture was prepared in PCR tubes by adding 0.5 µl of cDNA template, 1 µl of each of forward and reverse primers (PaLV 2430 F and PaLV 3561 R), 3 µl of Q5® Buffer, 0,3 µl of dNTPs, 0,2 µl of Q5® High-Fidelity DNA Polymerase and 9 µl of Milli-Q water.

Table 5. Gradient PCR amplification program.

Step	Temperature °C	Time	Number of Cycle
Initial Denaturation	98	30 s	1
Denaturation	98	10 s	40
Annealing	50 – 65	20 s	40
Extension	72	1 min	40
Final extension	72	2 min	1
Hold	4	∞	-

4.6.3 Virus specific RT-PCR

The presence of the virus was investigated in our samples. First, cDNA was prepared from our samples by reverse transcription, using the Revert Aid™ First Strand cDNA Synthesis Kit from total RNA template (as previously described), and then virus specific PCR reaction was performed at the optimal temperature using Q5® High-Fidelity DNA Polymerase.

Accordingly, the reaction mixture was prepared in PCR tubes by adding 0.5 µl of DNA template, 1 µl of each of forward and reverse primers (PaLV 2430 F and PaLV 3561 R), 3 µl of Q5® Buffer, 0,3 µl of dNTPs, 0,2 µl of Q5® High-Fidelity DNA Polymerase and 9 µl of Milli-Q water. Finally, the PCR program was performed in a thermal cycler according to the following table.

Table 6. RT-PCR amplification program

Step	Temperature °C	Time	Number of Cycle
Initial Denaturation	98	30 s	1
Denaturation	98	10 s	40
Annealing	65	20 s	40
Extension	72	1 min	40
Final extension	72	2 min	1
Hold	4	∞	-

4.6.4 Gel electrophoresis

In order to detect PaLV-specific targeted sequence, each of the RT-PCR products was mixed with 3 µl of DNA loading die and loaded into 1.2% agarose gel, besides a 100bp molecular weight ladder in the gel's first lane, to be separated by electrophoresis at 110 V. Gels were documented under UV light with a Bio-RAD chemidoc MP imaging system.

4.6.5 Purification of the PCR fragments

For sequencing purpose, the 1131 bp RT-PCR products of interest have to be purified from the agarose gel. For purification, the Thermo Scientific GeneJet Gel Exctraction Kit was used.

First, the PCR product was precisely excised from the gel, using a sterile scalpel, and placed into a previously weighed 1.5 ml microcentrifuge tube. Afterwards, the tube was weighed again to check the slice weight, and a binding buffer (1:1) was added accordingly for dissolving the gel, denaturing proteins, and to promote DNA binding to the column.

To make sure that the gel slice was completely dissolved, the tube was incubated at 60°C for 10 minutes. The resulted solution was transferred into the GeneJet purification column followed by centrifugation for 1 min and discarding the flow-through. Subsequently, 700 µl of Wash Buffer was added to the column, followed by centrifugation for 1 min and discarding the residual Wash Buffer.

Later, the column was placed into a new Eppendorf tube, and then 25 µl of elution buffer was added to the center of the column membrane followed by centrifugation for 1 min. Finally, the Eluted DNA was stored at -20 °C until used.

4.6.6 Cloning

The Thermo Scientific CloneJET PCR Cloning Kit was used for this purpose. The linearized cloning vector (pJET1.2/blunt) is able to take insertions of 6 bp up to 10 kb.

7,5 µl 2X Reaction Buffer, 0,75µl of the pJET1.2/blunt Cloning Vector (50 ng/µl), 0,5µl of T4 DNA ligase, 1µl of water nuclease-free and 5 µl of the DNA fragment were mixed to make a ligation mixture that has been added to a 1.5 ml Eppendorf tube. The tube was incubated at room temperature for 5 min then the ligation mixture was ready for transformation.

4.6.7 Transformation

The competent cells of *E.coli* (DH5 alpha strain) were stored at -70°C, and put on ice for 10-15 minutes to defrost. Afterwards, 200 µl of the competent cells were added to empty transformation tubes on ice, and mixed with 5 µl pf the ligation mixture, then stored on ice for 20 minutes. To make the heat shock effect, the tubes were subsequently moved to a 42°C water bath for 30 seconds and moved back on ice.

500 µl of SOC medium, without antibiotics, was added to the tubes before transferring them to a shaking incubator at 37°C for 40 minutes allowing the bacteria to recover and express the antibiotic resistance marker encoded in their plasmid. Afterwards, 250 µl of the transformed competent cells were transferred into an LB solid medium, containing ampicillin, and spread carefully.

The Petri dishes were inverted and incubated at 37°C overnight where the transformed colonies were estimated to appear within 12-16 hours.

4.6.8 Inoculation of liquid culture

250 ml of LB medium and 250 µl of ampicillin were added into an Erlenmeyer flask. Subsequently, the inoculation flasks were filled with 3 ml of that mixture. By using toothpicks, 4 colonies were individually collected from each of the overnight-incubated Petri dishes and subsequently used to inoculate a new Petri dish (filled with solid LB medium and labelled) by drawing a line using the same toothpick that was placed afterwards into its inoculation flask. Finally, the cultures were incubated overnight at 37°C inside a shaker.

4.6.9 Plasmid purification

The plasmid purification was performed using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL), where the 3 ml transformed *E.coli* cells were transferred into 1.5 ml Eppendorf tube followed by a centrifugation step, for 3 minutes at 8000 rpm, to sediment the cells. Afterwards, the supernatant was discarded, and 250 µl of Buffer A1 was added and pipetted up and down to completely re-suspend the pelleted cells.

Subsequently, 250 µl of Lysis Buffer A2 was added and mixed gently by inverting the tube 6 times. The tube was left 5 minutes at room temperature for incubation until the lysate was clear. Afterwards, 300 µl of Precipitation Buffer A3 was added and immediately mixed by inverting the tube until the mixture was homogeneous, followed by a centrifugation step at room temperature for 5 min at 11000 g.

The NucleoSpin® Plasmid column was placed in a collection tube where 750 µl of the supernatant were added followed by a centrifugation step for 1 min at 11000 g discarding the flow-through afterwards and placing the column again into the collection tube.

Finally, 600 µl of Buffer A4 was added to the column followed by a centrifugation step for 1 min at 11000 g. The empty column was placed back into the collection tube and later centrifuged for 2 min at 11000 g to dry the silica membrane. The NucleoSpin® Plasmid column was lastly placed into 1.5 ml microcentrifuge tube, mixed with 30 µl of Buffer TAE to elute the plasmid, and incubated for 1 minute at room temperature followed by a centrifugation step for 1 min at 11000 g.

4.6.10 Plasmid digestion

Xho1 and Xba1 (Thermo Fischer Scientific) are two restriction enzymes that were used to digest the pJET plasmids in order to make sure that the cloned PCR fragments of interest were successfully inserted into these plasmids.

2 µl 10X Tango Yellow Buffer, 0.2 µl of Xho1, 0.4 µl Xba1, 4.4 µl of Milli-Q water, and 3µl of the purified plasmid were mixed and incubated for 1 hour at 37°C. Subsequently, each of the purified undigested and the digested plasmids were separated by gel electrophoresis, and only those plasmids with the inserted fragment were sent for Sanger sequencing.

4.6.11 Analyzing of the sequences

4.6.11.1 Chromas 2.6.6

As described on its official website, Chromas software is a free trace viewer for simple DNA sequencing projects that do not require assembly of multiple sequences. Among the features that Chromas offers is the ability to export the sequences in many formats such as FASTA in order to use it for further analysis.

Chromas software has been used in this work to export the sequenced PCR products of Elberta, Champion and Suncrest varieties isolates as FASTA format in order to use it to build a phylogenetic tree using MEGA software.

4.6.11.2 BLASTn

The Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was used to pairwisely align the reference coat protein sequence of PaLV (NC_034970.1) with the sequences of Elberta, Champion and Suncrest PCR products that were obtained using the newly designed PaLV-coat protein specific primers (PaLV_F_2430 and PaLV_R_3561).

Furthermore, since the diagnostics lab had previously obtained the sequences of PaLV isolates from Champion and Suncrest peach mother trees (were kept inside the greenhouse), the coat protein sequences of Champion and Suncrest mother trees isolates were also pairwisely aligned, using BLASTn, with the sequenced PCR products of Champion and Suncrest varieties isolates.

4.6.11.3 Clustal omega

Clustal Omega is a multiple sequence alignment program, provided by the European Bioinformatics Institute (EMBL-EBI), that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. The three PaLV coat protein sequences originated from Champion, Elberta and Suncrest peach varieties were aligned using this program to find out any possible variations.

4.6.11.4 MEGA

The Molecular Evolutionary Genetics Analysis (MEGA) is a computer software, developed by the Pennsylvania State University, for conducting statistical analysis of molecular evolution and for constructing phylogenetic trees. It includes many sophisticated methods and tools for phylogenomics and phylomedicine (Wikipedia, 2019). MEGA has been used to build up a phylogenetic tree represents the evolutionary relationships among each of the reference isolates of PaLV (IVIM18 and Konela), peach varieties isolates (Champion, Elberta and Suncrest), and peach mother trees isolates (Champion and Suncrest).

5. RESULTS

5.1 RNA isolation

The attached figures (5 and 6) confirm a successful total RNA extraction from all of the samples of interest, where intact, sharp bands of 28S and 18S rRNA are shown in each of the corresponding gels.

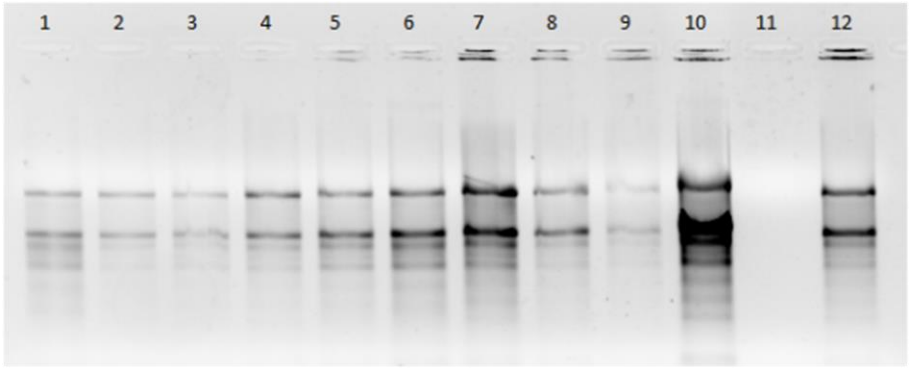

Figure 5. Extracted RNA of peach varieties leaf samples (the 11th lane repeated in the 13th and 14th lanes of the next figure).

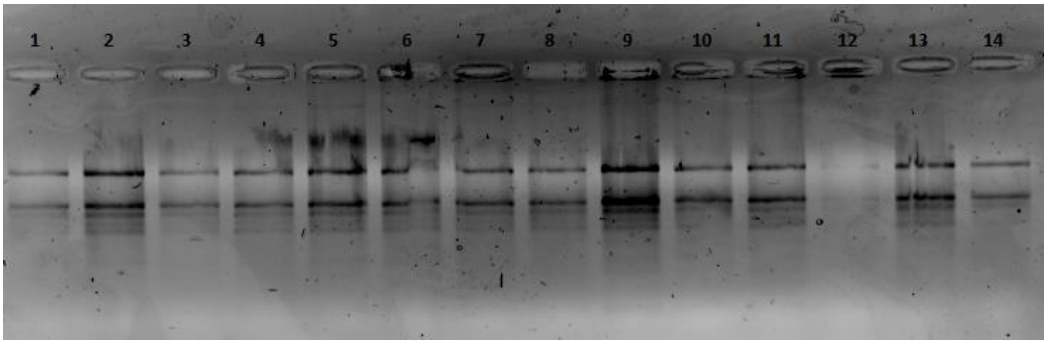

Figure 6. Extracted RNA of the woody indicators leaf samples (Both the 13th and 14th lanes correspond to the 11th peach variety leaf sample).

5.2 cDNA synthesis and quality test

The complementary DNA synthesis from total RNA samples, of both the varieties and the woody indicators leaf samples, was carried out using the Revert Aid™ First Strand cDNA Synthesis Kit. In order to

test the quality of the generated cDNA, an endogenous internal control (β-actin housekeeping gene) was utilized to perform a control PCR amplification with actin specific primers amplifying a 719 bp product from the endogenous peach actin gene. The following Figures (7 and 8) show a distinct 719 bp PCR product present in all of the investigated samples, which indicates a successful cDNA synthesis.

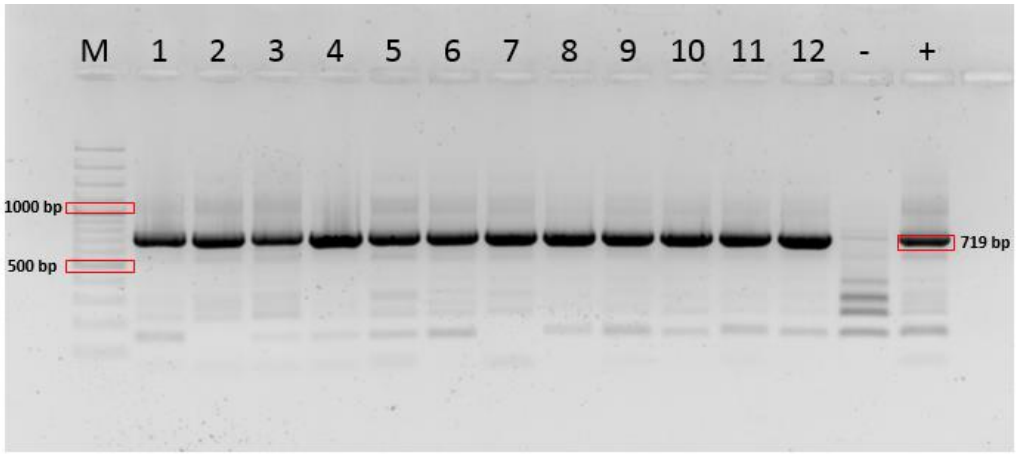

Figure 7. The intact and sharp 719 bp bands, amplified by PCR with β-actin specific primers, indicate a successfully synthesized cDNA of peach varieties leaf samples from their corresponding total RNA

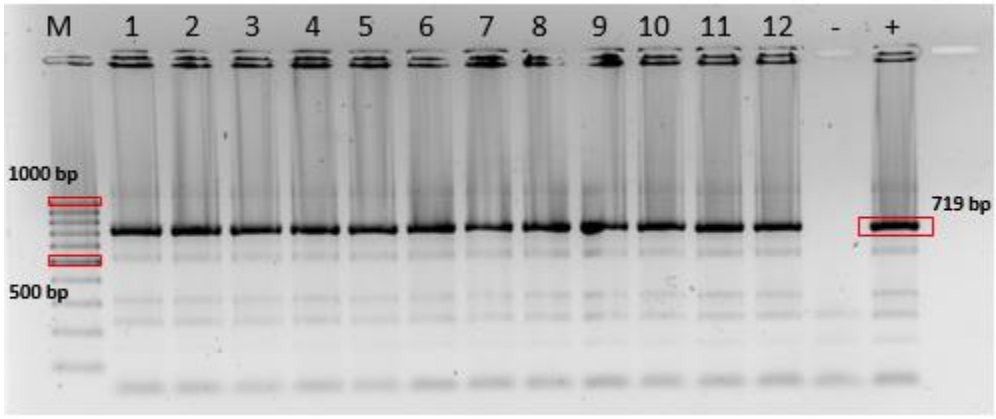

Figure 8. The intact and sharp 719 bp bands, amplified by PCR with β-actin specific primers, indicate a successfully synthesized cDNA of peach woody indicators leaf samples from their corresponding total RNA

5.3 Gradient PCR

The thermal gradient feature, available on thermal cyclers, allows identification of the best annealing temperature for the primer set in a single run. A gradient PCR was performed using a temperature gradient of 5 different annealing temperatures (ranged from 50°C to 65°C) to determine a single annealing temperature that will provide efficient, specific amplification of PaLV coat protein.

Figure 9. Results of the gradient PCR with PaLV_F_2430 and PaLV_R_3561 primers, indicate that 65 °C is the best annealing temperature for the designed primers (the 1000 bp band of the molecular wright ladder is marked with red rectangle).

5.4 Diagnostics of PaLV

To identify the presence of PaLV, PaLV_F_2430 and PaLV_R_3561 virus specific primers were used in the RT-PCR applied on the cDNA of both the peach varieties of interest and their corresponding woody indicators. As a positive control, a Reliable cDNA sequence from previous RTs was used whereas Milli-Q water was added instead of the cDNA template as a negative control.

5.4.1 PaLV in peach varieties

As the following figure shows, PaLV was detected as a 1131 bp product (the expected molecular weight of the amplified coat protein sequence) in Elberta, Champion, and Suncrest peach varieties (the 5th, 9th and 10th samples respectively).

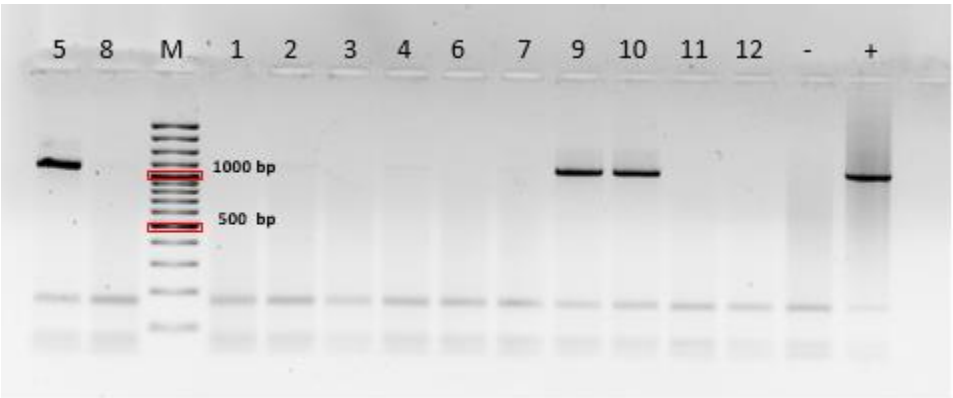

Figure 10. Results of the RT-PCR with PaLV_F_2430 and PaLV_R_3561 primers indicate the presence of PaLV as an expected 1131 bp band in each of Elberta, Champion and Suncrest peach varieties respectively.

5.4.2 In the woody indicators

According to the RT-PCR results, PaLV could not be detected in any of the woody indicators leaf samples, thus, raising the doubt of these woody indicators' actual capacity to reveal an occurred viral infection.

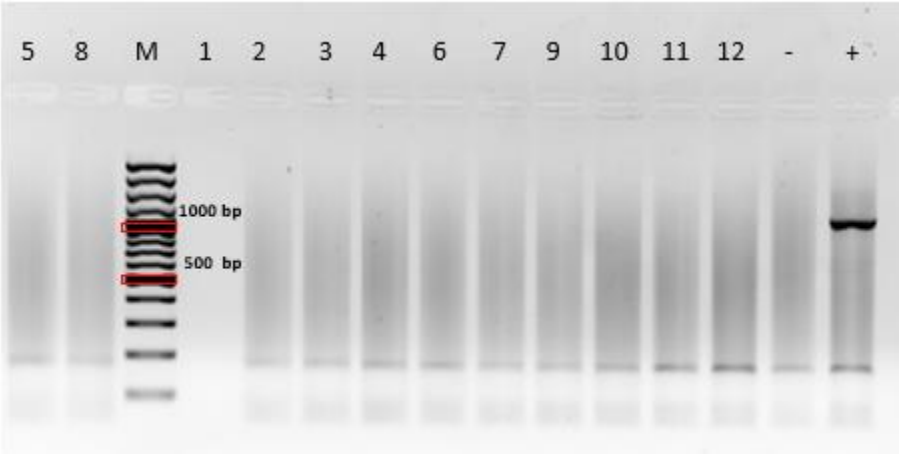

Figure 11. Results of the RT-PCR with PaLV_F_2430 and PaLV_R_3561 primers indicate the absence of PaLV in all of the sampled woody indicators.

5.5 Comparision of PaLV sequences to the reference genome using BLASTn

Table 7. BLASTn identities parameter comparing the coat proteins sequeces of PaLV reference sequence and each of Elberta, Champion and Suncrest peach varities isolates, as well as to compare the coat protien sequnces of Champion and Suncrest mother trees isolates with their corresponding Champion and Suncrest varieites isolates .

Coat Protein Sequence	Elberta variety isolate	Champion variety isolate	Suncrest variety isolate
PaLV reference (NC_034970.1)	976/988(99%)	954/968(99%)	932/968(96%)
Champion mother tree isolate	NA	909/911(99%)	875/911 (96%)
Suncrest mother treee isolate	NA	890/914(96%)	912/914(99%)

5.5.1 PaLV reference sequence with Elberta variety isolate sequence

MO_variety_5_Elberta
Sequence ID: Query_106653 Length: 988 Number of Matches: 1

Range 1: 1 to 988 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1759 bits(952)	0.0	976/988(99%)	0/988(0%)	Plus/Minus

Figure 12. Results of the pairwisely alignment of PaLV reference sequence with Elberta variety isolate using BLASTn.

5.5.2 PaLV reference sequence with Champion variety isolate sequence

MO_variety_9_Champion
Sequence ID: Query_152673 Length: 968 Number of Matches: 1

Range 1: 1 to 968 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1711 bits(926)	0.0	954/968(99%)	0/968(0%)	Plus/Minus

Figure 13. Results of the pairwisely alignment of PaLV reference sequence with Champion variety isolate using BLASTn.

5.5.3 PaLV reference sequence with Suncrest variety isolate sequence

MO_variety_10_Suncrest
Sequence ID: Query_131795 Length: 968 Number of Matches: 1

Range 1: 1 to 968 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1591 bits(861)	0.0	932/968(96%)	0/968(0%)	Plus/Minus

Figure 14. Results of the pairwisely alignment of PaLV reference sequence with Suncrest variety isolate using BLASTn

5.5.4 PaLV of Champion mother tree with PaLV of Champion variety

Champion_ D270_8
Sequence ID: Query_173457 Length: 1026 Number of Matches: 1

Range 1: 116 to 1026 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1672 bits(905)	0.0	909/911(99%)	0/911(0%)	Plus/Minus

Figure 15. Results of the pairwisely alignment of PaLV from Champion mother tree with the isolate of Champion variety using BLASTn

5.5.5 PaLV of Suncrest mother tree with PaLV of Suncrest variety

Suncrest_D270_9
Sequence ID: Query_214965 Length: 1024 Number of Matches: 1

Range 1: 111 to 1024 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1679 bits(909)	0.0	912/914(99%)	0/914(0%)	Plus/Minus

Figure 16. Results of the pairwisely alignment of PaLV from Suncrest mother tree with the isolate of Suncrest variety using BLASTn

5.5.6 PaLV of Suncrest mother tree with PaLV of Champion variety

Suncrest_D270_9
Sequence ID: Query_130817 Length: 1024 Number of Matches: 1

Range 1: 111 to 1024 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1500 bits(812)	0.0	880/914(96%)	0/914(0%)	Plus/Minus

Figure 17. Results of the pairwisely alignment of PaLV from Suncrest mother tree with the isolate of Champion variety using BLASTn

5.5.7 PaLV of Champion mother tree with PaLV of Suncrest variety

Champion_ D270_8
Sequence ID: Query_68357 Length: 1026 Number of Matches: 1

Range 1: 116 to 1026 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1485 bits(804)	0.0	875/911(96%)	0/911(0%)	Plus/Minus

Figure 18. Results of the pairwisely alignment of PaLV from Champion mother tree with the isolate of Suncrest variety using BLASTn.

5.6 Multiple comparision of PaLV sequences using Clustal Omega

The three PaLV nucleotide sequences originated from Champion, Elberta and Suncrest peach varieties were aliened using Clustal Omega program to find out any possible differences. The results of percent identity matrix, generated by Clustal2.1, indicate that Elberta and Suncrest isolates are the most homogenous.

```
MO_variety_10_Suncrest      CTGAGGAGCTGCGTCTACCTAGCAAGGTCCTTGGAAATTCACATTGAAGGAGATAACGAA      60
MO_variety_5_Elberta        CTGAGGAGCTGCGTCTACCTAGCATGGTCCTTGGAAATTCACGTTGAAGGAGATAACGAA      60
MO_variety_9_Champion       CTGAGGAGCTGCGTCTACCTAGCATGGTCCTTGGAAATTCACGTTGAAGGAGATAACGAA      60
                            ************************ ***************** ******************

MO_variety_10_Suncrest      TTGGCCCGCGATGTCCGACGACTTCCCGTTTCCTTTGTAGACAAGCCAGAATTGATCCTC      120
MO_variety_5_Elberta        TTGGCCCGCGATGTCCGTCGACTTACCGTTTCCCTTGTAGACAAGCCAGAATTGATCCTC      120
MO_variety_9_Champion       TTGGCCCGCGATGTCCGTCGACTTACCGTTTCCCTTGTAGACAAGCCAGAATTGATCCTC      120
                            ***************** ****** ******** *************************

MO_variety_10_Suncrest      CGAAGTGGGGTGCCAGAGGAGTCCACGGAGGACTCGGGCCTGGAACCCACGAGAGAATCC      180
MO_variety_5_Elberta        CGAAGTGGGGTGCCAGAGGAGTCCACGGAGGACCCTAGCCTGGAACCCTCGAGAGAATCC      180
MO_variety_9_Champion       CGAAGTGGGGTGCCAGAGGAGTCCACGAAGGACCCTTGCCTGGAACCCTCGAGAGAATCC      180
                            *************************** ***** *  *********** ***********
```

Figure 19. The multiple alignment of PaLV isolates from Suncrest, Elberta and Champion peach varieties using Clustal Omega. Stars in the figure refer to conservative regions among the three isolates.

Percent Identity Matrix - created by Clustal2.1

```
1: MO_variety_10_Suncrest  100.00   96.69   96.28
2: MO_variety_5_Elberta     96.69  100.00   99.17
3: MO_variety_9_Champion    96.28   99.17  100.00
```

5.7 MEGA

MEGA software was used to build up a phylogenetic tree represents the evolutionary relationships among each of PaLV reference isolates (IVIM18 and Konela), peach varieties isolates (Champion, Elberta and Suncrest), and peach mother trees isolates (Champion and Suncrest).

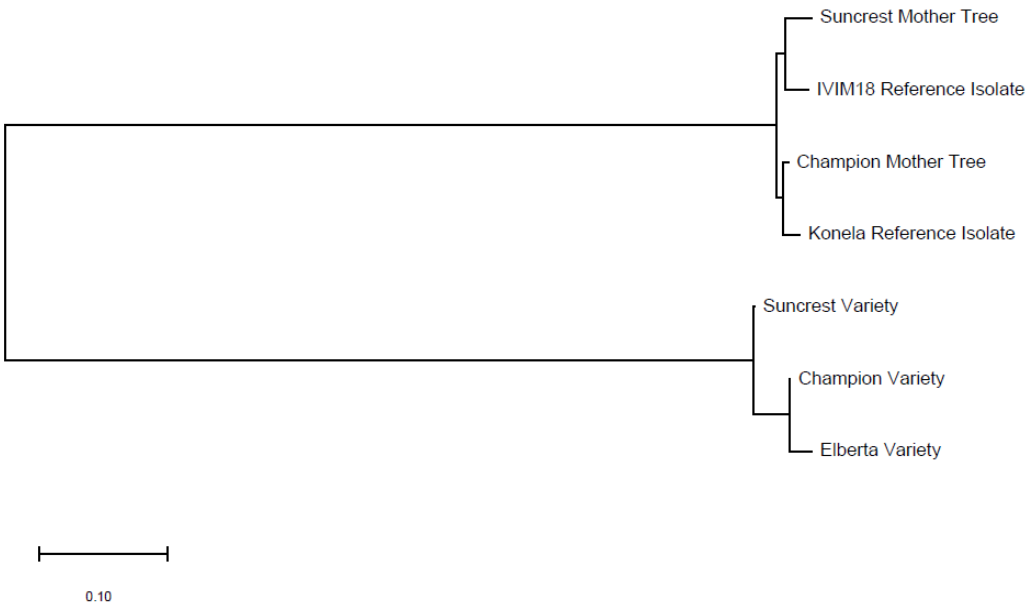

Figure 20. A phylogenetic tree, generated by MEGA software, represents the evolutionary relationships among each of PaLV reference sequences, peach varieties PaLV isolates and peach mother trees isolates.

As the 3 PaLV isolates of peach varieties, were grown in field conditions, clustered together independently from which variety they have been isolated from, it is possible to conclude that the PaLV infection might has happened in the field and was not transmitted from the mother trees by grafting.

6. CONCLUSIONS AND RECOMMENDATIONS

During our work, we tested the presence of PaLV, a new, emerging virus in Hungary in peach samples. The virus was first detected in the mother trees by small RNA HTS. Our RT-PCR results showed that the virus could be detected in the grafted varieties, but not in the woody indicators of the biotest. In addition, the generated phylogenetic tree raised the question of the evolutionary relationships among the three sequenced isolates from the peach varieties in the field conditions and two previously sequenced isolates from peach mother trees in the greenhouse conditions.

1- PaLV was detected by RT-PCR, in 3 out of 12 tested peach varieties, validating its presence that was originally described using small RNA-HTS by the Diagnostics group of NARIC.

2- PaLV could not be detected by RT-PCR in any of the 12 tested woody indicators supporting the notion that indexing on woody indicators could be problematic for new, emerging viruses.

3- The phylogenetic tree that has been created from the sequenced PaLV strains showed that the three isolates of PaLV, derived from peach varieties in the field conditions, have made an independent cluster from the two isolates derived from peach mother trees in the greenhouse conditions. What therefore suggest that the PaLV infection has happened most probably in the field conditions and not because of grafting of the improperly virus released variety.

4- PaLV is a new, emerging virus in Hungary and it would be important to build a whole picture on their etiology, biology and epidemiology. Nevertheless, knowing the economic consequences of hosting such viruses in peach trees is the core goal in order to put this in practice when it comes to quarantine measures.

7. SUMMARY

As vegetatively propagated plants, peach trees can be infected by more than 20 different viruses and viroids. PaLV was among many novel viruses that have been identified in peach trees using HTS analysis over the last years, the technique that has revolutionized viruses' discovery and identification.

The main goal of this investigation was to compare the efficiency of biotest indexing with the molecular detection (RT-PCR), to test peach varieties that were cultivated in a fruit trees nursery. As the Diagnostics team of NARIC has detected PaLV in the tested mother trees and validated the results of the performed HTS analysis, during this work we tested the presence of this virus in the woody indicators and the grafted varieties.

In the course of this investigation, primers were newly designed according to the small RNA reads that were generated during small RNA HTS in order to specifically amplify the PaLV coat protein. Afterwards, the PCR products were purified, cloned and sequenced by Sanger method. Finally, the sequenced PCR products have been analyzed using different bioinformatics tools, such as Chromas, BLASTn, Clustal omega and MEGA software.

Indeed, the results of BLASTn analysis showed that the PCR products were the PaLV coat protein, therefore, the presence of PaLV could be detected in 3 out of 12 tested peach varieties and the results of the small RNA HTS platform have been confirmed. On the other hand, the negative PCR results in 12 out of 12 tested woody indicators raise the doubts over the sensitivity of biotest indexing for detecting new, emerging viruses.

Furthermore, using OMEGA software, the generated phylogenetic tree has addressed the question of the evolutionary relationships between the three isolates of PaLV, derived from the peach varieties, and both of previously obtained mother trees isolates. The results provided us with an indication that the peach varieties isolates were evolutionary more related than the mother trees isolates that have shown relevance with the PaLV reference sequences, what could possibly explain that the peach varieties have been infected in the field conditions rather from the use of infected plant material during the grafting procedure.

8. ACKNOWLEDGEMENTS

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DECLARATION

Me, as the undersigned Mohammad Omran (Code-Neptun: DMEDC2) declare, that the Diploma Thesis entitled Molecular Detection of Peach-associated Luteovirus (PaLV) in Peach Trees submitted on 29th of April, 2019 is my own intellectual property.

I hereby acknowledge that the presentation of my thesis in the Dean's Office according the schedule does not mean at the same time the acceptance of my dissertation from professional and content related aspects.

, Budapest