Hungarian University of Agriculture and Life Sciences

PhD Thesis

Virus diagnostics of peach and apricot cultivars with next-generation sequencing

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Gödöllő

2021

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INTRODUCTION AND OBJECTIVES

Apricot and peach orchards are affected by several fungi, bacteria, virus and viroid pathogens. Viral infection can reduce crop quantity and quality, however visual symptoms are not present in the case of latent infections. The prevention of infection is the best way to keep the plantations healthy in the case of viruses. Using virus free propagation material and regular monitoring of mother trees with sensitive virus diagnostic methods have pivotal role in defense against pathogens.

Quarantine pathogens are screened with biological indexing, PCR and ELISA assays, but we are able to detect all of pathogens within the investigated samples applying metagenomic approaches. Even more pathogen is detected from those countries, where the presence of them was not described before.

Sequencing of small RNAs is a special technique of high-throughput sequencing. RNA interference is a conserved, eukaryote specific defense mechanism against exogenous nucleic acids. During viral replication double stranded RNAs are produced, which are cleaved into 21-24 nt long small interfering RNAs (siRNA) by the RNase activity of DICER

enzymes (Csorba & Burgyán, 2016). With the help of small RNA HTS, we are able to obtain comprehensive knowledge about distribution and host plants of viruses or viroids.

During our research we performed small RNA HTS examinations from apricot and peach trees of Fruitculture Research Institute.

Objectives:

1. Optimise small RNA HTS method for virus diagnostics of apricot and peach samples.
2. Determine the virome of examinated individuals, emphasizing the investigation of those viruses, which were not described in Hungary before.
3. Draw conclusion to the source of infection from the virus profile of isolator houses and stock nurseries.
4. Compare the efficiency of small RNA HTS and biological indexing as diagnostic methods.
5. We would determine different strains of identified viruses in order to examinate the applicability of diagnostic PCR primers for Hungarian virus variants as well.

MATERIALS AND METHODS

The collected apricot and peach samples derived from the isolator houses and stock nurseries of NARIC Fruitculture Research Institute of Érd. Ligeti óriás, Pannónia, Magyar kajszi apricot and Springcrest, Cresthaven peach samples were collected from isolator houses and stock nurseries. Further 10 cultivars (Flavortop, Nektár H, Venus, Incrocio Pieri, Cresthaven, Redhaven, Champion, Suncrest, Aranycsillag, Apolka) were selected to our research, because these cultivars were under biological indexing examination by the employees of Virological Station of National Food Chain Safety Office.

RNA extraction from leaves was performed by modified CTAB-based protocol (Gambino et al., 2008). Individual RNA samples were pooled equal concentration to obtain more information about the virological status of trees. In the case of apricot 6 RNA pool were prepared from Ligeti óriás, Pannónia and Magyar kajszi cultivars, which were derived from isolator house and stock nursery. 4 pool were prepared from isolator house and stock nursery derived Springcrest and Cresthaven peach cultivars and one another pool was created from 10 cultivars of peach isolator house (Flavortop, Nektár

H, Venus, Incrocio Pieri, Cresthaven, Redhaven, Champion, Suncrest, Aranycsillag, Apolka).

Small RNA libraries were prepared from RNA pools using optimized Illumina Truseq Small RNA Library Preparartion Kit (Czotter et al., 2018). Small RNA fraction was isolated from total RNA extract, then 3' and 5' adapter sequences were ligated and reverse transcription was performed. After PCR amplification, the small RNA fraction was separated by polyacrylamide gel electrophoresis. During bioinformatic analysis of small RNA libraries small RNA sequences and contig sequences were aligned to the RefSeq database of NCBI. The positive virus result should fulfil the following requirements:

- the virus specific non-redundant and normalised redundant (redundant read count / 1000000) read count exceeds the threshold of 200 reads,
- presence of matching contig
- minimum 40% coverage of reads to the virus reference genome.

Verification of bioinformatic results was performed by RT-PCR with published and self-designed primers, and Northern-blot in the case of CVA, PaLV and PLMVd. In

order to determine the whole sequence of PCR products we cloned them into pJET1.2/blunt vectors. For the multiple alignment analysis of PCR product and clone sequences Clustal Omega and for phylogenetic analysis MEGA 6.0 software was applied.

RESULTS

Small RNA HTS results of apricot trees

Cherry Virus A (CVA), Little cherry virus 1 (LChV-1) and Plum pox virus (PPV) were identified with small RNA NGS from apricot samples. CVA infection was verified from Pannónia cultivar with RT-PCR and Northern-blot. All of 5 Pannónia individuals and 2 *in vitro* were CVA infected. Phylogenetic analysis showed, that the apricot derived clone sequence grouped together with non-cherry host CVA isolates, especially with other apricot derived isolates from Canada and Japan.

Regarding RT-PCR amplification of HSP70h and coat protein, one of two stock nursery derived Magyar kajszi samples showed positive result for LChV-1. The Hungarian LChV-1 strain has showed close phylogenetic relationship with American and Czech isolatos from apricot host and from Italian sour cherry derived strain, which is responsible for Kwanzan stunting syndrome. Comparison analysis of CP and HSP7h regions of Hungarian LChV-1 sequences revealed high similarity with Madarska and Magiar kajszi cultivars from Czech Republic. PPV was detected from stock nursery

of Ligeti óriás and Magiar kajszi cultivars. In the case of Magyar kajszi cultivar PPV and LChV-1 coinfection was confirmed.

Small RNA HTS results of peach trees

Peach-associated luteovirus (PaLV), *Nectarine stem-pitting-associated virus* (NSPaV) and *Peach latent mosaic viroid* were detected from peach small RNA libraries.

PaLV infection was identified from the following libraries: Springcrest isolator house and stock nursery, Cresthaven stock nursery and 10 cultivars comprising peach isolator house library. The occurrence of PaLV was validated by amplification of 1132 bp long ORF3a, coat protein and movement protein encoding region with RT-PCR. During investigation of individuals 3 of 4 Springcrest isolator house samples and 1 of 2 stock nursery samples and both *in vitro* samples were PaLV infected. 2 Cresthaven isolator house derived samples and 4 samples (Cresthaven, Champion, Suncrest, Arancsillag) showed positive PCR and Northern-blot results from 10 cultivars representing isolator house pool.

NSPaV was detected from isolator house and stock nursery of Springcrest, stock nursery of Cresthaven, and from 10 cultivars representing isolator house pool. In order to validate bioinformatic resukts, 1752 bp long coat protein and read-through protein encoding region of NSPaV was amplified with RT-PCR. All 4 individuals of Springcrest isolator house pool and both *in vitro* plants were infected, while 1 of 2 individuals of Cresthaven stock nursery pool gave positive result for NSPaV.

PPV was detected from Springcrest isolator house and stock nursery pools, which was confirmed with amplification of coat protein.

Cresthaven isolator house and stock nursery derived libraries were positive for PLMVd, furthermore all of investigated individuals were infected according to RT-PCR and Northern-blot verification. RT-PCR primers were able to amplify the whole viroid genome, and radioactively labelled probe also matched to the entire viroid sequence. 6 cultivars were infected with PLMVd from 10 peach cultivar representing pool. During analysis of clone sequences from Cresthaven isolator house and stock nursery the vast majority of point mutations were localised in the loops of viroid

structure, which do not possess pivotal role to maintain the stability. More small nucleotide polymorphism pairs were identified, which did not affect the viroid's secondary structure because the changed nucleotides have kept complementarity.

Significant tree losses were observed in the peach isolator house, therefore extended virologic testing was performed with RT-PCR based on HTS virus results. From 89 peach trees 65 was virus or viroid infected. In 19 cases PaLV, 17 cases NSPaV, 15 cases PPV and 59 cases PLMVd was identified. During phylogenetic analysis cultivar specific clustering of PaLV sequences was observed from individuals of Michelini, Springcrest and Cresthaven cultivars, while other cultivars' trees grouped separately. The cloned coat protein sequences of NSPaV clustered separately from the sequences of GenBank database, however the comparison of partial coat protein sequences resulted a monophyletic group between NSPaV sequences from Szob and Érd.

PLMVd sequences showed significant genetic variability within individual trees too. A 12-nucleotide long insertion was detected within Öb166/1 cultivar derived PLMVd sequence. The insertion does not show any sequence

similarity with peach calico resulting insertion despite the common localization on the viroid genome. Each one of 11 cultivars (Flavortop, Nektár H, Venus, Incrocio Pieri, Cresthaven, Redhaven, Champion, Suncrest, Apolka, Aranycsillag, Elberta) were under biological indexing examination at Virological Laboratory of National Food Chain Safety Office whitin the period of 2016-2019. PLMVd specific visual symptoms were not visible on grafted GF305 indicators, despite the positive HTS, PCR and Northern-blot results of the grafts' mother trees. PLMVd transmission from scion to GF305 indicator was verified in the case of Venus, Incrocio Pieri, Cresthaven, Champion, Suncrest, Elberta and Apolka cultivars. PLMVd was detectable from every single GF305 indicator, which were derived from viroid infected mother trees.

New scientific results

1. Based on bioinformatic analysis of small RNA HTS and verification results I concluded that small RNA HTS is also a powerful virus diagnostic method in the case of apricot and peach samples.
2. The presence of PaLV and PLMVd was described at first in Hungary. CVA and LChV-1 were detected

from apricot, which were undescribed in Hungary before. Our group was among the first to describe apricot as a host of LChV-1.

3. The presence of PaLV and PLMVd was described at first in Hungary.
4. We concluded propagation material derived infection, because of the presence of CVA, LChV-1, PaLV, NSPaV and PLMVd in isolator house.
5. Despite of the large extent of PLMVd infection in peach isolator house, viroid could not be solely responsible for tree decay.

CONCLUSIONS

Because of technical improvement and reduction of charges, metagenomic approaches are getting even more widespread in the field of virus diagnostics.

Our group successfully optimised small RNA HTS-based virus diagnostic protocol for woody plants. In the case of investigation of 5 apricot libraries CVA, LChV-1 and PPV was detected. CVA was identified from Pannónia isolator house and stock nursery and LChV-1 from Magyar kajszi stock nursery library. We detected CVA and LChV-1 at first time from Hungary. Our group was among the first to describe apricot as a host of LChV-1. Presumably CVA infection occurred via propagation material derived manner, because CVA was also detected from isolator house, stock nursery and *in vitro* samples while LChV-1 infection occurred in open field, because only from stock nursery was the virus detectable. Phylogenetic analysis revealed the adaptation to host plants in the case of CVA and LChV-1. Based on these results the host plant species has major impact on homology, than the geographical origin of examined viruses. PPV infection occurred in open field, because the virus was detectable only from stock nursery.

During analysis of peach small RNA libraries PaLV, NSPaV, PPV and PLMVd were detected. NSPaV and PPV have already been described from Hungary, but PaLV and PLMVd were undescribed yet.

PaLV infection happened via propagation material derived manner, because Springcrest isolator house, stock nursery and *in vitro* samples were positive for PaLV. The source of NSPaV cannot be determined, because in the case of Springcrest cultivar only the isolator house, but in the case of Cresthaven only the stock nursery sample was infected. Unfortunately, we do not have enough information about symptom development and economic impact of PaLV and NSPaV, because these can be considered newly discovered viruses.

PPV was detected from Springcrest isolator house, stock nursery and *in vitro* samples, PLMVd was identified from Cresthaven isolator house and stock nursery. The presence of these pathogens in isolator house and stock nursery confirms the possibility of propagation material derived infection. Both PPV and PLMVd are quarantine pathogens, which are regulated within the meaning of decree14/2017. (III.23.).

During analysis of Cresthaven isolator house and stock nursery derived PLMVd sequences 11 point mutation pair were localised on the viroid structure, which did not affected the base pairing of opposite nucleotides, therefore the viroid is able to maintain its characteristic branching conformation.

We observed remarkable tree decay in the peach isolator house. In order to get a more comprehensive picture about the virological status of isolator house PaLV, NSPaV, PPV were tested with RT-PCR and PLMVd with Northern-blot. We could not investigate the perished trees and some of dying trees were not virus infected, therefore the exact cause of decay remained uncleared. Even though the high virus/viroid infection rate presumably other pathogens could be responsible for peach decay in isolator house.

During sequence analysis of PLMVd 12 nucleotide insertion have been found from Öb166/1 cultivar, which localized at same position as the insertion resulting peach calico phenotype. The Öb166/1 PLMVd clone did not show homology with peach calico insertion, therefore the produced small RNAs from this region do not lead to the degradation of mRNAs of HSP90, which is a main factors within chloroplast biogenesis.

PaLV, NSPaV and PLMVd were detected from peach isolator house, but on the indicators did not appeared virus specific symptoms. Our examinations proved the transmission of PLMVd from graft to indicator, however the infection remain latent in the vast majority of cases, therefore indicator plant-based virus diagnostics can lead to false negative result.

PUBLICATIONS

Scientific articles in reviewed journals with impact factor

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