Theses of PhD dissertation

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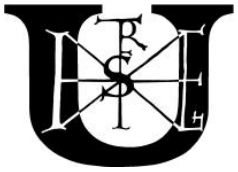
Gödöllő

2019



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Molecular investigation of gene-expression changes that play a key role in the development of disease symptoms in virus-infected plants

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Gödöllő

2019

Doctoral school

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THE BACKGROUND AND OBJECTIVES OF THE STUDY

In compatible plant-virus interaction, the virus alters the host plant metabolism in order to replicate its genome and spread cell to cell in the plant (systemic infection). Based on the severity of these changes we can distinguish two types of virus infections. In case of acute infection, the virus presents in large concentration, severe disease symptoms appear which can even lead to the death of the plant within a short time. During persistent virus infection, the virus is present also in large concentration, but the symptoms on the host are milder and the plants survive.

Plant viruses can inhibit the expression of certain genes through the regulation of host messenger RNA (mRNA) (shut-off phenomenon). Our group has shown that: (1) some viruses are capable of reducing the expression of a host's housekeeping genes, while other viruses are unable to reduce the expression of a host's housekeeping genes, (2) the shut-off manifests itself in the nucleus at the level of transcription and (3) there may be a relationship between the intensity of the symptoms and the extent of the shut-off. Subsequently, our group performed microarray analysis to monitor genome-wide gene-expression changes in virus-infected *Nicotiana benthamiana* plants. The evaluation of the microarray analysis was the beginning point of my work.

The aims of our study were to explore the effects of virus infection on the host plant gene-expression system and to understand the processes behind these changes and their role in the development of the symptoms.

The aims of my PhD work were:

1. To investigate CymRSV, crTMV, TCV infected *Nicotiana benthamiana* and PVX, TMV infected *Solanum lycopersicum* plants by two different high throughput methods (microarray hybridization and RNA sequencing).
2. To confirm the changes in gene-expression by different methods (Northern blot, qRT-PCR).
3. To investigate the presence of shut-off on TMV, PVX and TRV virus vectors (VIGS) infected *N.benthamiana* and *S.lycopersicum* plants by confirming expression of the *Rubisco*, *Gapdh* and *tubulin* genes.

MATERIAL AND METHOD

Virus strains

The *Nicotiana benthaminana* plants were infected with crucifer infecting tobacco mosaic virus (crTMV), Cymbidium ringspot virus (CymRSV, Cym19stop), turnip crinkle virus (TCV), carnation Italian ringspot virus (CIRV, CIRV19stop). tobacco mosaic virus U1 strain (TMV-U1) and potato virus x (PVX) were used to infect the *Solanum lycopersicum* plants.

Infection of the test plants, sample collection

For infection, we used *in vitro* transcripts of the viruses or purified virions, to which celite-containing inoculating buffer and water (1:10 ratio) were added. The infection was mechanically performed with the glass spatula.

Nicotiana benthamiana plants were infected at 4-5 leaf stage (plants were 15-20 days) with crTMV, CymRSV and TCV virus and virus-free inoculation buffer (mock) for microarray analysis validation. We collected systemically infected leaves at 5 days post inoculation (dpi) from CymRSV and crTMV infected plants and 11 dpi from TCV infected plants.

Solanum lycopersicum (Kecskemét jubileum) plants were infected with TMV and PVX virus and virus-free inoculation buffer (mock) after the first leaf was developed (plants were 19-20 days) for RNA-seq and further validation. We collected systemically infected leaves at 14 dpi from TMV and PVX infected plants.

In each case we collected the samples from mock-inoculated plants on the same time. Our experiments were performed in three biological replicates of both plants.

RNA sequencing

RNA sequencing was used to more precisely monitor gene-expression changes. Total RNA extract was prepared from virus-infected *S.lycopersicum* plants by phenol-chloroform method and we pooled the total RNA extracts and the samples were purified with RNazol.

Purified RNAs were sequenced with UD-GenoMed Ltd. on the Illumina HiScanSQ NGS platform. RNA sequencing of samples was performed from three biological replicates.

RESULTS

The shut-off is not a consequence of necrosis

In our investigations, we wanted to exclude the possibility that the shut-off phenomenon is caused by necrosis. As a proof of *N.benthamiana* plants were infected with wild-type (CymRSV, CIRV) and p19 deficient mutant viruses (CymRSV19stop, CIRV19stop).

The infected plants were grown at 15°C. Under low-temperature conditions the virus spreads more slowly in CymRSV and CIRV infected plants, the virus can also accumulate to a high level in Cym19stop and CIRV19stop infected plant without necrosis. We have been showed by Northern blot that the expression of *Rubisco* and *CP29* genes decreased in CymRSV, CIRV and Cym19stop, CIRV19stop infected plants versus the control. *PR-Q* expression induced in both virus infections.

Effect of virus infections on host plant gene-expression system

The results of the microarray analysis and RNA sequencing showed that the gene-expression changes were more drastic in acute infections (CymRSV, crTMV, PVX) than in persistent infections (TCV, TMV). We have been showed that the number of downregulated genes was much higher than the upregulated genes in *N.benthamiana* plants.

Functional categorization of genes with significant expression

We functionally grouped genes that significant gene-expression changes in any virus infection compared to control. We have been showed that virus infections strongly influenced the expression of the genes involved in photosynthesis, cell wall metabolism, stress, RNA regulation, protein metabolism, signal transduction, cell cycle and transport in both host plants.

Stress genes

Based on microarray analysis and RNA sequencing results, in case of acute infection the expression of stress genes was drastically altered, most of the genes were induced, whereas in persistent infection the expression of stress genes remains unchanged in both host plants. We confirmed by Northern hybridization, that the expression of *PR1*, *PR-Q*, *SAR* and *GST* genes in *N.benthamiana* and the expression of *GST* and *HSP20* genes in *S.lycopersicum* plants was induced in acute infections whereas expression of these genes remained unchanged in persistent infection.

Genes involved in cell wall metabolism

Our results of the microarray and RNA sequencing showed in both host plants the expression of genes involved in cell wall metabolism decreased during the acute infections, in contrast these gene-expression did not change in persistent infections. We have been showed by high throughput methods that in acute infections the levels of the *CESA8* were downregulated, whereas the *CWINV2* were upregulated which plays a key role in cellulose synthesis. The findings were validated by qRT-PCR.

Genes involved in photosynthesis

We have been shown that during acute infection, the expression of genes involved in photosynthetic processes was reduced in tobacco and tomato. In contrast the expression of these genes was not altered in persistent virus infection. We showed by Northern blot analysis that expression of *PAO* gene was induced during acute (CymRSV and crTMV) infections, whereas in persistent infection the *PAO* expression in tobacco remains unchanged. RNA sequencing results in *S.lycopersicum* showed *PAO* expression slightly decreased in PVX and slightly induced in TMV infection.

Investigation of key regulators that influence gene-expression

Based on microarray analysis and RNA sequencing results, the expression of the *BZL4*, a *NAC-like*, *ZFP19* and *WRKY70 transcription factor*s induced, but the *LRR-containing transmembrane kinase* and *TMKL1* expression decreased in acute virus-infected tobacco and tomato plants. The expression of these genes was not or only slightly altered in persistent virus-infected plants.

In our studies we demonstrated that the expression of *AGO4* and *methyltransferases* was reduced in both host plants during the acute infection. Northern blot analysis on tobacco plants confirmed that *AGO4* expression was decreased in CymRSV and crTMV virus infection, whereas in TCV virus infection the *AGO4* expression did not change.

Investigation of the shut-off phenomenon

Based on microarray analysis and RNA sequencing, the expression of important housekeeping genes reduced, it showed shut-off phenomenon, in acute virus-infected tobacco and tomato plants. We confirmed by Northern blot that the expression of *Rubisco*, *Gapdh*, *CP29* gene reduced in acute virus-infected tobacco and tomato plants, whereas these genes-expression remains unchanged in persistent infection. We confirmed by qRT-PCR that expression of *EF* and *histone* genes reduced in PVX infected tomato plants.

We demonstrated by Northern blot that GSyV1 and GPGV viruses did not cause a reduction in the expression of investigated genes (*Rubisco*, *EF*, *actin*) in *Vitis vinifera* plants.

In addition we confirmed by Northern blot analysis that the expression of the most commonly used housekeeping genes (*tubulin*, *Gapdh*, *Rubisco*) altered in TMV-, PVX- and TRV VIGS infected *N.benthamiana* and *S.lycopersicum* plants.

New scientific results

1. I have been confirmed that the decrease of the expression of endogenous genes (shut-off) during the virus infection was not the result of the necrosis.
2. Based on the analysis of the microarray results of the virus-infected *Nicotiana benthamiana* plants, I found that the acute CymRSV and crTMV infection caused more drastic gene-expression changes at the genomic level than persistent TCV infection.
3. I have verified the microarray results by Northern hybridization or qRT-PCR and I proved that in case of CymRSV and crTMV infection the expression of protective genes (*PR1, PR-Q, SAR, GST*) and the *CWINV2* gene were drastically induced, while the expression of housekeeping genes (*Rubisco, Gapdh, CP29, AGO4, PAO*) and *CESA8* gene were decreased, as opposed to TCV infection, where expression of these genes remained unchanged.
4. I have been showed by RNA sequencing of virus-infected *Solanum lycopersicum* plants that acute PVX infection caused seriously gene-expression changes as compared to persistent TMV infection.
5. I have been confirmed the RNA sequencing results by Northern hybridization or qRT-PCR and I proved that in case of PVX infection the expression of stress genes (*HSP20, GST)* and *CWINV2* gene were induced, while the expression of housekeeping genes (*Rubisco*, *Gapdh*, *CP29, EF*, *histone*) and *CESA8* gene were decreased, as opposed to TMV infection, where expression of these genes remained unchanged.
6. Summarizing the above results, I pointed out the significant gene-expression change and shut-off phenomenon can be detected only in acute infection in the investigated *Nicotiana benthamiana* and *Solanum lycopersicum* plants.
7. I found that the shut-off did not occur on the expression of housekeeping genes in case of GSyV1 and GPGV infected grapevine plants.

CONCLUSIONS AND SUGGESTIONS

Our results show that both high throughput methods (microarray and RNA sequencing) are suitable of tracking gene-expression changes in compatible plant-virus interactions. The changes in expression levels were higher in the case of microarray analysis compared to RNA sequencing, which could be a result of different detection methods. The ratio of downregulated genes in microarray analysis was higher compared to that in upregulated ones, which could be a result of the presence of overrepresented probes.

We investigated the effect of acute and persistent infections on gene-expression in two different host plants and we observed significant differences. 1) We have been shown that the acute virus infections cause drastic while persistent virus infections cause minimal gene-expression changes in tobacco and tomato. 2) We have verified that the stress genes were induced in acute infections but these genes induction were absent in persistent infections. 3) Our group demonstrated that alteration of key regulators of RNA interference is also observed only in acute virus infections. This may explain the reduction in gene-expression of many genes during the acute virus infection and that in the absence of changes in the key regulators of RNA interference, the metabolism of the host remains untouched in persistent infections.

We have revealed the expression of the possible genes involved in photosynthetic processes, leaf senescence (*PAO*), stunting, chlorosis and yellowing (*CESA* and *CWINV)* changed drastically in acute infection and simultaneously these plants showed more drastic symptoms.

The shut-off did not occur on the expression of housekeeping genes in GSyV1 and GPGV infected grapevine plants. The reason for this may be that the longer period of infection process and the viruses are also present at a lower level in woody plants than in herbaceous plants.

The expression of the most commonly used for qRT-PCR housekeeping genes (*tubulin*, *Gapdh*, *Rubisco*) altered in TMV-, PVX- and TRV VIGS

infected *N.benthamiana* and *S.lycopersicum* plants. Therefore, the effect of virus vectors on gene-expression must be checked and the housekeeping genes used as a reference value should be carefully selected before using the VIGS vector.

APPEARED PUBLICATIONS IN THE SUBJECT OF THE DISSERTATION

International, reviewed scientific publications

Pesti R., Kontra L., Paul K., Vass I., Csorba T., Havelda Z., Várallyay É. (2019): Differential gene expression and physiological changes during acute or persistent plant virus interactions may contribute to viral symptom differences. PLOS ONE 14(5): e0216618. https://doi.org/10.1371/journal.pone.0216618

Oláh E., **Pesti R.**, Taller D., Havelda Z., Várallyay É. (2016): Non-targeted effects of virus-induced gene silencing vectors on host endogenous gene expression. Archives of virology 161(9): 2387-93 p. DOI: 10.1007/s00705-016-2921-9

National, reviewed scientific publications

Czotter N., Szabó E., Molnár J., **Pesti R.**, Oláh E., Deák T., Bisztray Gy., Tusnády E. G., Kocsis L., Burgyán J., Várallyay É. (2015): Szőlőültetvényeink metagenomikai diagnosztikája új, hazánkban eddig nem leírt vírusok jelenlétét mutatta ki. Növényvédelem 51(12):550-558 p.

International conferene publications

Pesti R., Kontra L., Kenny P., Molnár J., Tusnády E. G., Vass I., Havelda Z., Várallyay É. 2017. Characterization of gene expression and physiological changes in different host-virus interactions. Hungarian Molecular Life Sciences, Eger, 2017.02. 04-03.31., ISBN 978-615-5270-34-5

Pesti R., Oláh E., Kagan F., Havelda Z., Várallyay É. 2017. Sequence requirement of viral suppressor mediated miR168 induction in plant. Hungarian Molecular Life Sciences, Eger, 2017.02. 04-03.31., ISBN 978-615-5270-34-5

Pesti R., Havelda Z., Várallyay É. 2015. Gene expression changes behind symptom development in virus infected plants. Hungarian Molecular Life Sciences**,** Eger, 2015.03.27-29., ISBN 978-615-5270-15-4

National conferene publications

Pesti R., Kenny P., Vass I., Havelda Z., Várallyay É. 2016. A vírusfertőzés tüneteinek kialakulásában szerepet játszó génexpressziós változások vizsgálata. Növényvédelmi Tudományos napok, Budapest, 2016.02.16-17., ISSN 0231 2956

Pesti R., Molnár J., Kenny P., Vass I., Tusnády E. G., Havelda Z., Várallyay É. 2016. Génexpressziós változások vizsgálata vírusfertőzött paradicsomban. FIBOK, SZIE, Gödöllő, 2016.03. 21-22. ISBN 978-963-269-536-5

Oral and poster presentation

Pesti R., Molnár J., Kenny P., Vass I., Tusnády G. E., Havelda Z., Várallyay É. 2016. Characterization of gene expression and physiological changes in different host-virus interactions. AAB Conference: International Advances in Plant Virology, Greenwich, 2016.09.07-09.

Pesti R., Kontra L., Havelda Z., Várallyay É. 2017. Lehet-e a kis RNS-eknek szerepe a shut-off-ban? RNS szalon, NAIK-MBK, Gödöllő, 2017.06.23.

Pesti R., Kenny P., Kontra L., Molnár J., Tusnády E. G., Vass Imre, Havelda Zoltán, Várallyay Éva 2016. Akut és perzisztens vírusfertőzés hátterében álló molekuláris változások. MBK Napok, NAIK-MBK, Gödöllő, 2016.12.14-15.

Pesti R., Molnár J., Kenny P., Papp-Kádár V., Vértessy B., Vass I., Tusnády E. G.,Marincs F., Havelda Z., Várallyay É. 2015. Növény válaszreakciói vírusfertőzés hatására. MBK Napok, NAIK-MBK, Gödöllő, 2015.11.11-13.

Science book, book chapter, book editing

Czotter N., Molnár J., **Pesti R.**, Demián E., Baráth D., Varga T., Várallyay É. (2018): Use of siRNAs for Diagnosis of Viruses Associated to Woody Plants in Nurseries and Stock Collections. Methods Mol Biol. 1746:115-130 p. doi: 10.1007/978-1-4939-7683-6_9. PubMed PMID: 29492890.

Science Education (TDK Consultant)

Kagan Ferenc - Virális géncsendesítést gátló fehérjék által indukált miR168 promóter analízise tranziens génexpressziós rendszerben. ELTE Növénytudományi Szekció. Témavezetők: Dr. Várallyay Éva, **Pesti Réka**