Final report of NKFIH (OTKA) funded project K108718

Molecular mechanism behind disease symptom development in virus infected plants

Successful viral infection and development of disease symptoms are mainly determined by the efficiency of virus spread in the plant and the magnitude of virus induced changes in the host metabolism. Movement and expression changes depends on the type of the plant-virus connection. In the K108718 project we aimed to investigate the complex regulation pattern of AGO1, the master component of RNA silencing machinery, its role in symptom development and in defence reactions against different viruses. Furthermore, we wanted to analyse the additional role of other, not AGO1 connected, gene-expression changes in the determination of the severity of disease symptoms. We planned this project for four years and planned the research to carry out by my PhD students: Enikő Oláh and Réka Pesti. As they were both PhD students for three years with a scholarship, we could save some Personnel money and extend the project for an additional year. This spring Enikő Olah defended her PhD thesis: "VIGS VEKTOROK HASZNÁLATA SORÁN BEKÖVETKEZŐ GÉN EXPRESSZIÓS VÁLTOZÁSOK ÉS EGY VIRÁLIS GÉNCSENDESÍTÉST GÁTLÓ FEHÉRJE RNS KÖTÉSÉNEK VIZSGÁLATA". We have just finished the manuscript summarizing our results about gene-expression studies in acute and persistent virus infected plants. This published research will be the base of Réka Pesti's PhD thesis. However this manuscript is currently under review and not yet accepted for publication why we attached it in a ready to publish format the end of this report.

During the planned project we had to main objectives:

1. Revealing the complex molecular mechanism behind the miR168 mediated AGO1, the key regulator for RNAi, regulation.

In the proposed research we planned to prove that enhanced accumulation of miR168 is directly correlated to the presence of different unrelated viral silencing suppressors (VSRs) with transient expression study. Moreover, as the common feature of the VSRs is the ability to bind siRNAs, using mutant p19 VSR, we wanted to answer the question whether siRNA binding deficient mutant p19 VSR is able to induce miR168 accumulation and consequently control of AGO1 level, and how will this mutant VSR interferes with the development of disease symptoms? Both of our studies were finished after the preparation of the proposal but before the beginning of this new OTKA fund and were accepted as two full paper article with the acknowledgement of my PD OTKA: Éva Várallyay and Zoltán Havelda (2013): Unrelated viral suppressors of RNA silencing mediate the control of ARGONAUTE1 level . Mol Plant Pathol. 2013 6:567-75. and Éva Várallyay, Enikő Oláh and Zoltán Havelda (2014): Independent paralleled functions of p19 plant viral suppressor of RNA silencing required for effective suppressor activity Nucleic Acid Research 42:599-608.This is why on this field we focused on other aspects of the regulation.

Analysis of reporter elements responsible for induction of miR168 and AGO1 mRNA

To reveal the molecular mechanism laying behind the regulation of AGO1, GFP based sensor test system was created. The miR168 target site with its neighbouring sequence from both *A. thaliana* and *N. benthamiana* and *L. esculentum* was cloned into a GFP containing sensor constructs both into translatable (in frame with GFP), and also in non-translatable region. With the sensor constructs transient expression studies were carried out. In order to find important regulators of VSR induction we analysed the promoter by bioinformatics methods for the presence of regulatory elements. In parallel miR168a promoter fragments with different lengths were cloned into the GFP containing

binary vector. Using these constructs minimal lengths of both *A. thaliana* and *S. lycopersicum* miR168a promoters induced by p19-3M were determined and compared. Different unrelated VSRs can induce miR168 level also. In this transient expression system minimal promoter requirement for this effect using different VSRs was also investigated. Changes in both in the GFP mRNS and miR168 miRNA level was confirmed by Northern hybridization. To test the promoter activity not only in transient assays, but also in virus infected plants *N.benthamiana* was transformed with the whole and the truncated miR168a promoter. These experiments served as a base for an MsC thesis (Kuglics Alexandra – Riporter rendszer kidolgozása az ARGONAUTE' miR168 általi szabályozásának vizsgálatára dohányban) and a Scientific student studies (Kuglics Alexandra -TDK 1st prize at ELTE Biology, 2013 nov, Kagan Ferenc, TDK ELTE Biology 2016 and OTDK 2017). Preliminary results about the promoter analysis were summarized and presented as a poster at 3rd Hungarian Molecular Life Sciences Conference (Pesti et al: Sequence requirement of viral suppressor mediated miR168 induction in plants). As the preliminary results seems ambiguous we decided not to continue this part of the project. However the *N.benthamiana* transgenic lines will be available for use in the future if we would like to test the miR168 induction ability of new silencing suppressors.

Mimicking AGO1 down regulation in miR168 overexpressing *A. thaliana*

To dissect the role of AGO1 down regulation from other gene expression changes induced during viral replication we planned to mimic the virus induced down regulation of AGO1 by over expressing miR168 in *A. thaliana*. To do this *A. thaliana* was transformed with Arabidopsis *MIR168* a and b. In the second year we reached T5 generation. Although overexpression of miR168 was uniform, its regulation effect on AGO1 protein level manifested only in inflorescent and in young leaves. Investigating the level of another AGO (AGO2 protein) by western blot, showed only slight increase in inflorescence and a presence of a new isoform, suggesting that beside AGO2 another AGO – possible AGO5 and AGO10 can be further involved to rescue the plant in the absence of AGO1. In our transgenic lines only slight correlation of the level of miR168 with symptoms were found. As in our preliminary work we have found that only small portion of miR168 is in a protein complex further investigated this question. We have generated artificial miRNA precursors expressing miR168 (but from a different miRNA precursor structure). We have found that the structure of the precursor seems to be very important and can be a key element of the regulation process. We have generated artificial miRNA precursors expressing miR168 (but from a different miRNA precursor structure) and investigated this question in transgenic mir168 overexpressing lines by gel filtration. We have found that balanced loading efficiency of miR168 into ARGONAUTE1 is determined by the secondary structure of the precursor RNA. The extent of miR168 action is regulated mainly through its AGO1 loading rate, and this can be modulated by making alterations in MIR168 precursor. We have found that both the structure of the miR168/miR168* duplex and the precursor backbone affect the loading efficiency of miR168 into AGO1. The enhanced loading of miR168 resulted in elevated AGO1 down regulation and delayed flowering. These data suggest a novel link between miRNA biogenesis and RISC loading mediated by RNA structure of the miRNA precursor.

These results were showed on a poster at LIFE2017 conference, and in a talk at the Genetics Miniconference and RNA Salon by Dalmadi Ágnes. A manuscript was prepared: Precursor RNA structure mediated balanced loading of miR168 regulates ARGONAUTE1 homeostasis, summarizing our results so far with the above abstract:

"ARGONAUTE1 (AGO1) is the main executor component of the RNA-induced silencing complex (RISC) predominantly responsible for micro RNA (miRNA) mediated repression of target mRNAs. *AGO1* mRNA itself is also under the control of miRNA pathway through miR168. In previous studies we revealed the presence of unusually high level of free, protein unbound miR168. Here we show that miR168 over-expression from wild type stem-loop of miR168a primary transcript results mainly in the accumulation of free miR168 species and only the minority of miR168 excess is loaded

into the AGO1/RISC, indicating that miR168 is regulated rather through its AGO1 loading rate than its biogenesis. We demonstrate that this loading equilibrium could be shifted towards AGO1 incorporation by the expression of miR168 from artificial stem-loops having heterologous structural features, resulting enhanced AGO1 down-regulation and delayed flowering. In *dcl1* and *drb1* mutants where production of most miRNAs, are severely reduced AGO1 level is also down-regulated via enhanced AGO1 loading of miR168. These data suggest, that AGO1/RISC homeostasis is under the control another type of miR168 mediated regulation, where the unbound pool of efficiently matured miR168 is present in excess and structural elements of the precursor define its balanced loading rate into free AGO1/RISC through competition with other small RNA species. Our data also suggest a novel link between miRNA biogenesis and RISC loading mediated by the RNA structure of the miRNA precursor."
However we realized that for finishing this work we need some further experiments which we plan to finish till the end of this year.

2. Elucidation of the molecular mechanisms determining symptom development in different plant-virus interaction.

In my previous PD OTKA project a microarray experiment was carried out to characterize gene-expression changes in virus infected *N. benthamiana*. During this project we wanted to characterize gene-expression changes in virus infected *S. lycopersicum* by RNA seq. All of these experiments were carried out. The two methods on these host were compared and validated by Northern blot experiments or by qRT-PCR. Moreover in a collaboration with HAS Biological Center at Szeged physiological characterization of both *N.benthamiana* and *S.lycopersicum* were carried out. Based on the gained results a manuscript was prepared: Comparison of Gene Expression and Physiological Changes in Acute and Persistent Plant Virus Infections with the above abstract and is currently under review (see the edited manuscript at the end of the report.)

During the project results of this work presented at different conferences by Réka Pesti – who was the main researcher working on this project.

At FIBOK206 Conference Réka won a third prize with her talk (Pesti Réka és mtsai: Génexpressziós változások vizsgálata vírusfertőzött paradicsomban and at an International Advances in Plant Virology, Conference of AAB in Greenwich Pesti et al: Characterization of gene expression and physiological changes in different host-virus interactions, she won the student prize award. We participated in a joint project on *N.benthamiana* sequencing which resulted in a paper (Baksa et al: Identification of *Nicotiana benthamiana* microRNAs and their targets using high throughput sequencing and degradome analysis. BMC Genomics. 2015 Dec 1;16(1):1025.)

Although the main conclusion of the manuscript is that we cannot show the mechanism how viruses in persistent infection can avoid to interfere with their host we tried to find some answer. According to our previous studies shut-off develops in the nucleus of the infected cell, but molecular mechanism of it is still not know. As uracil in the DNA can serve as signal for different expression of genes during different condition we have checked the possibility that is it possible that the presence of shut-off is regulated through this mechanism. We have purified genomic DNA from virus infected tomato and uracil content of our samples were tested in a collaboration with Beáta Vértessy at Research Center of Natural Sciences of HAS. Unfortunately investigation of uracil level in virus infected plants showing shut-off and not

showing shut-off compered to mock inoculated plants have not shown differences suggesting that some other type of mechanism is involved.
Réka also cloned promoters of GAPDH and Rubisco from *N.benthamiana* and carried out methylation experiments but during that we faced lots of technical problems. Later from the analysis of small RNA pattern of the virus infected plants (gained from small Illumina sequenced small RNA libraries) turned out that it is possible that methylation changes not only in the promoter but in the coding regions of these genes happens, why in the future we plant to also investigate this possibility.

Analysis of the small RNA reads from these samples added new aspects for regulation processes, why we purified small RNA fractions from virus infected *N.benthamiana* and *S.lycopersicum* and sequenced them. Analysing these data by bioinformatics methods we got interesting results: Investigation of the size distribution profile of the small RNA reads from PVX infected tomato showed that the level of 22nt long reads increased, probably because of the increased activity of DCL2. To fully analyse this question we infected wild type and a dcl2 mutant *N.benthamiana* with TMV and PVX, the same strains as we used on tomato, in 3 biological replicates. From the small RNA analysis of tomato samples we thought that small RNAs generated from housekeeping genes can have profound importance during persistent host gene downregulation. The NGS data was analysed by bioinformatics methods and now we started to validate the changes by small RNA Northern blot. These work was summarized by a talk at an RNA Salon meeting by Réka Pesti.
Réka has finished her PhD training and as soon as this work will be accepted for publication she will start the defence period, what will hopefully finished in early next year.

Our studies on shut-off raised the question how viral based VIGS vectors effect the host gene expression in plants and does it interfere with the results gained with this method. *N.benthamiana, S.lycopersicum* and *T.aestivum* was infected with TMV, PVX, TRV and BSMV based VIGS vectors. Expression level of reference genes (Rubisco, GAPDH, actin and elongation factor) was analysed applying northern blot. According to our results the expression of the widely used reference genes was almost always drastically down regulated. Our results suggest the necessity of careful selections of both genes, to be investigated and used as a reference control in the planned experiments.

VIGS studies were carried out by Oláh Enikő, who was my PhD student at SZIE.
We made a survey using different hosts and VIGS vectors to show some possible limit of this RNAi based high throughput technique. Investigation of *N. benthamiana, S. lycopresicum, T. aestivum* and *C. annuum* infected with different virus vectors (TMV, PVRX, TRV and BSMV), which are used in VIGS studies was completed and finished. Our manuscript summarizing these results was accepted and in the Archives of Virology (Archives of Virology 161:2387–2393). From the gained results Enikő Oláh prepared and defended her PhD thesis.

During a new project of our group investigating grapevine and fruit tree plantations we have found the presence of new viruses in Hungary. As these viruses are not characterized molecularly it is possible that they could serve as a base of a new VIGS vector. We investigated the presence of shut-off in grapevine infected with these viruses by Northern blot analysis and have found that mRNA level of actin, elongation factor and Rubisco was not altered – raising a possibility that these new viruses can serve as a base for VIGS vector development. This results was published in Czotter et al Növényvédelem 2015.

The same method, small RNA NGS, was used to survey fruit tree plantations and we have found the presence of not described viruses also. However this work not closely related to this NKFIH topic this work was published with the help of this project (we acknowledged this participation in the paper: Barath et al, Viruses 2018).

As a summary we can conclude that K108718 NKFI project helped us to reveal gene-expression differences during acute and persistent virus infection.
With the help of the project 3 papers in IF Journals were published (Archives of Virology, BMC Genomics and Viruses) and we will finish and publish more:
i/about miR168 regulation – the manuscript will be finished till the end of this year,
ii/about gene-expression studies (find the enclosed manuscript what is currently under review).

Moreover the project helped Enikő Oláh and Réka Pesti during their PhD work, Alexandra Kuglics for MSc thesis and Ferenc Kagan for TDK work.

International Journal of Molecular Sciences

MDPI

Article

Comparison of gene expression and physiological changes in acute and persistent plant virus infections

Réka Pesti[1], Levente Kontra[1], Kenny Paul[2], Imre Vass[2], Zoltán Havelda[1] and Éva Várallyay[1]*

[1] Agricultural Biotechnology Research Institute, NARIC; H-2100 Gödöllő, Szent-Györgyi A. St. 4., Hungary; pesti.reka@abc.naik.hu; kontra.levente@abc.naik.hu; havelda.zoltan@abc.naik.hu

[2] Biological Research Centre Szeged, HAS; H-6726 Szeged, Temesvári krt. 62., HungaryAffiliation 2; drkennypaul@gmail.com; vass.imre@brc.mta.hu

* Correspondence: varallyay.eva@abc.naik.hu; Tel.: +36 28 526140

Received: date; Accepted: date; Published: date

Abstract: Viruses have different strategies for infecting their hosts. Acute infection is fast resulting in severe symptoms or in the death of the plant, while in persistent interaction the virus can survive within its host for a long period of time with mild symptoms. During our studies we investigated gene expression changes of virus infected *Nicotiana benthamiana* and *Solanum lycopersicum* after systemic spread of the viruses by two different high-throughput methods: microarray hybridization or RNA sequencing. Using these techniques we could easily differentiate between acute and persistent infection and validate key gene-expression changes by Northern blot hybridization or qRT-PCR. Gene expression changes in hormone metabolism and other key regulation pathways during these different host-virus relationship can explain differences in the detected physiological changes and observed symptoms. We further show that by characterizing the photosynthetic activity of virus infected plants type of infection can be distinguished even at an early point. We demonstrate that in persistent infection not only drastic down-regulation of important housekeeping genes, but induction of stress genes is missing. Although it is still elusive how viruses can avoid interfering with their host this makes possible for them to survive for a long period of time.

Keywords: virus infection, gene-expression, shut-off, microarray, RNAseq, chlorophyll fluorescence, acute, persistent, *N. benthamiana*, *S, lycopersicum*

1. Introduction

Gene expression studies in virus infected plants usually investigate events at early time points after the infection just after the virus entered into the host cell. At this early point host antiviral responses are activated and the fate of the infection is determined. In non-host interaction the virus cannot replicate. If the plant is a host of the virus replication in the infected cell could happen, but thanks to the efficient resistance mechanisms most of the infections are confide to the infected cell and spread of the virus is blocked (reviewed recently [1]). In susceptible plant-virus interaction the virus gets over this barrier and steps into the next stage of the infection when finally it moves not only from cell to cell but also for long distances and colonize the whole plant. During this process other types of defence responses, like antiviral RNA interference (RNAi), are activated [2]. Viruses can block RNAi mechanisms by expressing viral suppressor proteins to inhibit RNAi and be able to reach a higher virus titre [3]. RNAi connected molecular mechanisms and their potential role in disease induction are well described (reviewed [4]), however changes in gene expression pattern of the host at this later step of infection, are still not fully characterized. With the availability both host genomes and high-throughput methods like microarray and RNAseq more and more studies

investigate this question and try to characterize molecular changes behind symptom development usually in a particular host-virus combination [5-16].

During virus infection the virus alters the host metabolism in order to replicates its genome and spread cell to cell in the plant. Susceptible host can be sensitive or tolerant for the particular virus. In sensitive host the infection is usually acute: the virus accumulates to high level and cause down-regulation of prominent housekeeping genes (shut-off) [17]. This can be linked to severe disease symptoms, which can even lead to the death of the plant within a short time. As a contrast in tolerant host the infection is persistent, the virus is presents also in large concentration, but not induce obvious symptoms, thereby the plant survive.

In our previous study we observed that some plant-virus interactions are able to decrease the expression of the important housekeeping genes and this "shut-off" persist for several weeks [18]. In this work we not only investigated gene expression pattern of some housekeeping genes, but using in vitro run on transcription experiments showed that the shut-off manifests in the nucleus at a transcription level [18].

In this report we describe gene expression changes both in *N. benthamiana* and in *S. lycopersicum* at a deeper insight. Both plants were infected by viruses causing persistent or acute infection. High-throughput methods: microarray or RNAseq were used to investigate gene-expression changes of the host at the genome level. Although we still cannot answer the question what is the molecular mechanism behind the "shut-off" mechanism, we could see and verify important commonalities and differences between persistent and acute infections and could correlate these changes with their physiological characteristics.

2. Results and discussion

2.1 Persistent down-regulation of housekeeping genes is not a consequence of necrosis

Virus infection can have profound effect on gene-expression pattern of the host. As a consequence, in acute plant-virus interactions, down-regulation of some important housekeeping genes (shut-off) occurs which can lead to the death of the plant or persists for several weeks in systemically infected leaves [18]. However, it is very difficult to differentiate if serious changes, appeared in shut-off presenting interaction, are causes or consequences of the further necrosis. Tombusviruses (like Cymbidium ringspot virus (CymRSV) and carnation Italian ringspot virus (CIRV)) infect *N. benthamiana* systemically and the infection culminates very fast into the death of the host. They encode a very efficient viral suppressor protein of RNA silencing (VSR), p19, which binds virus derived small interfering RNA (siRNA) duplexes in a size specific manner [19], and blocks both their loading into RNA induced silencing complexes and defence reactions of the plant [20]. However, in virus infection by p19 VSR deficient mutant viruses (CymRSV19S and CIRV19S) the antiviral system of the plant can work efficiently and after a recovery, almost healthy phenotype develops. In these plants, because of the efficient RNAi processes, low level of intact viruses are present. Efficiency of RNAi decreases at lower temperature [21], where during 19S mutant virus infection higher virus level can be reached. To exclude that housekeeping gene down- regulation is a consequence of early necrosis infection studies with p19S mutant viruses, inducing no necrosis, were carried out at 15°C. *N. benthamiana* plants were infected by wild type (CymRSV and CIRV) and p19 deficient mutant (CymRSV19S and CIRV19S) viruses and these plants were maintained at 15 °C. Leaf samples from newly developed leaves (systemic leaves) were collected at 12dpi and the expression level of two housekeeping genes, which play role in early photosynthetic processes: Rubisco, chlorophyll a/b binding protein 29 gene (CP29) , and one stress gene: pathogenesis related protein Q gene (PR-Q) were investigated by Northern blot. (SFigure 1). Similarly to GAPDH and tubulin, which level in CymRSV19S was reduced in our previous studies [18], Rubisco and CP29 level was down-regulated while the expression of the PR-Q was induced during virus infection irrespective of the presence of the suppressor. These expression changes were similar in wild type and mutant virus infection which never leads to necrosis, suggesting that shut-off is not a consequence of this process.

2.2 Sample preparation for high-throughput gene-expression analysis

In our previous study we observed that CymRSV, Crucifer infecting tobacco mosaic virus (crTMV) showed very severe symptoms on *N. benthamiana* and finally culminated in death of the plant. Potato virus x (PVX) showed intense chlorosis on *S. lycopersicum*, but necrosis never occurred and plants survived the infection. These infections were very fast and intense down-regulation of Rubisco and GAPDH could be detected as typical gene-expression changes for acute infection. In contrast Turnip crinkle virus (TCV) infected *N. benthamiana* and Tobacco mosaic virus U1 (TMV) infected *S. lycopersicum* show very mild symptoms of slight chlorosis. Neither Rubisco nor GAPDH levels were down-regulated, which is typical for persistent infection [18]. During this study our aim was to investigate gene-expression pattern of these hosts in both acute and persistent of infection in more detail. For the high-throughput characterization of the induced changes samples were collected from systemic leaves of virus infected plants, and the extracted RNA was used in our further experiments. *N. benthamiana* was infected with CymRSV, crTMV and TCV. Virus infection was carried out in three independent experiments on 10 plants/virus/biological replicates. Samples were collected at 5 dpi in case of CymRSV and crTMV infection and at 11dpi in case of TCV infection together with the appropriate mock inoculated samples (SFigure 2/a). At this time the virus could systemically infect the whole plant, leading to high virus level, but with moderate symptoms: stunting and mild chlorosis in each cases. Efficiency of virus infection was checked by virus specific Northern blot (SFigure 2/b). As a pilot reaction level of endogenous GAPDH was also checked by Northern blot (SFigure 2/c). RNA extracted from 10 plants/infection in 3 biological replicates/virus were used for microarray experiments. Three different varieties (Kecskeméti jubileum, M82, Moneymaker) of tomato were infected with TMV and PVX. Samples from symptomatic leaves (SFigure 3/b) were collected at 14 dpi. Endogenous Rubisco and GAPDH level was monitored by Northern blot to check how similar changes can be seen in different varieties (SFigure 3/c). Rubisco and GAPDH expression showed the same trends in all three varieties. For further studies Kecskeméti jubileum (a Hungarian variety) was selected and samples collected at 14 dpi. 4 plants/virus/biological replicates were used for RNAseq (SFigure 3/a). Virus level before pooling was checked by Northern blot (SFigure 3/d). In total RNAseq was done on 12 libraries (mock/PVX/TMV in 3 biological replicates, one of the biological replicates was sequenced twice as technical replicates).

2.3 Transcriptome profiling of virus infected N. benthamiana

Microarray analysis of CymRSV, crTMV, TCV and mock infected *N. benthamiana* were carried out as described above. Hierarchical clustering of the gained raw data showed that the biological replicates clustered together (SFigure 4/a). Moreover, results from persistent infection (TCV infected samples) clustered together with mock samples and distantly from samples originating from acute infection (CymRSV and crTMV infected samples), suggesting that changes in TCV infected *N. benthamiana* are more similar to non-infected samples than to changes in CymRSV and crTMV infected plants. To find differentially expressed genes, we collected all of the probes, which showed 2x changes and had p value smaller than 0.05 in any of virus infected plants (STable 1). Volcano plots of the detected changes clearly show that while in CymRSV and crTMV infected plants number of differentially expressed probes (DEPs) are high, this number in TCV infected samples are much less (Figure 1/a).

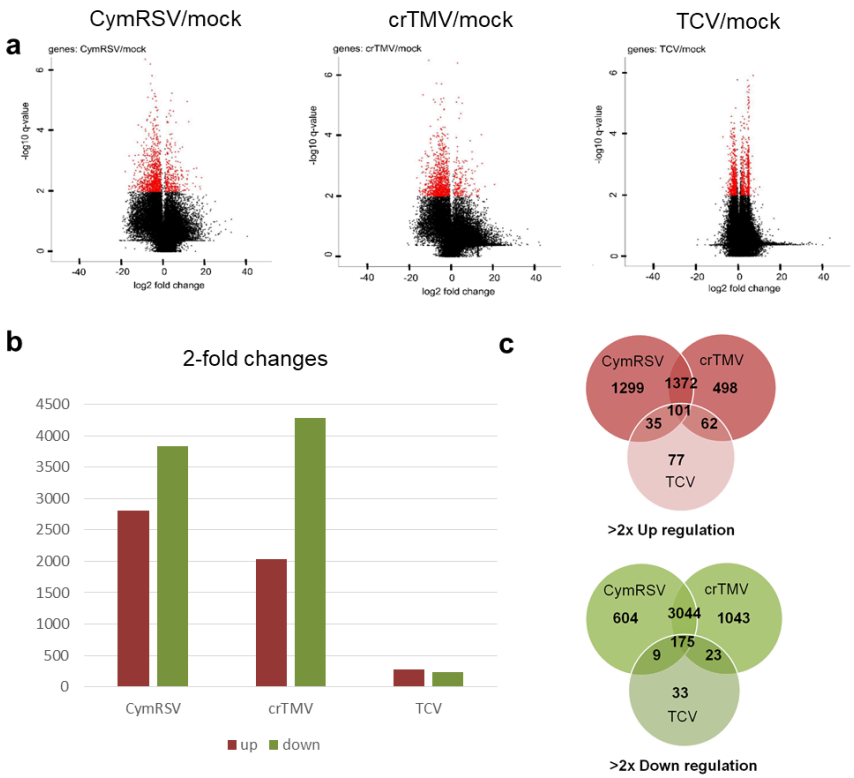


Figure 1. Gene expression changes in virus infected *N. benthamiana*
To identify differentially expressed probes each virus infected sample was compared to mock treated sample. Obtained fold changes were converted to log2 to generate volcano plots. More than 2-fold changes and p values less than 0.05 were applied to identify DEPs
a/ Volcano plots displays log2 fold changes and p values. b/ Column diagram of number of DEPs showing 2-fold changes. c/ Venn diagram of DEPs identified in the virus infected samples

In CymRSV, crTMV and TCV infected plants 6639, 6318 and 515 DEPs were identified, from which 2807, 2033 and 275 were up- and 3832, 4285 and 240 were down-regulated, respectively (Figure 1/b). Venn diagrams of the differentially expressed probes highlight that majority of the up- (1473) and down-regulated (3219) probes were similar in CymRSV and crTMV infected plants (Figure 1/c). These results show that while in acute infection gene-expression pattern of the host changed severely, this change affects fewer genes with a smaller extent in persistent infection. Although there are differences between the altered genes during two acute infections (CymRSV and crTMV), the majority of changes affect the same genes suggesting some common regulation.

2.4 Gene-expression changes in virus infected S. lycopersicum

RNAseq analysis of the virus infected *S. lycopersicum* was carried out in three biological and one technical replicates. Hierarchical clustering of the raw data showed that technical replicates (mock2 and mock3, PVX2 and PVX3 and TMV2 and TMV3) always clustered together confirming that the sequencing was reliable (SFigure 4/b). PVX samples clustered together and distantly from mock and TMV samples. Surprisingly, mock0 clustered with PVX samples, but this could happen due to some abiotic changes in the environment during the first experiments. To find differentially expressed genes (DEGs), which showed 2x changes and had p value smaller than 0.05 during virus infection were collected (STable 2). Volcano plots of DEGs (twofold changes with a p value lower than 0.05) show that more changes could be detected in PVX than in TMV infected samples (Figure 2/a).

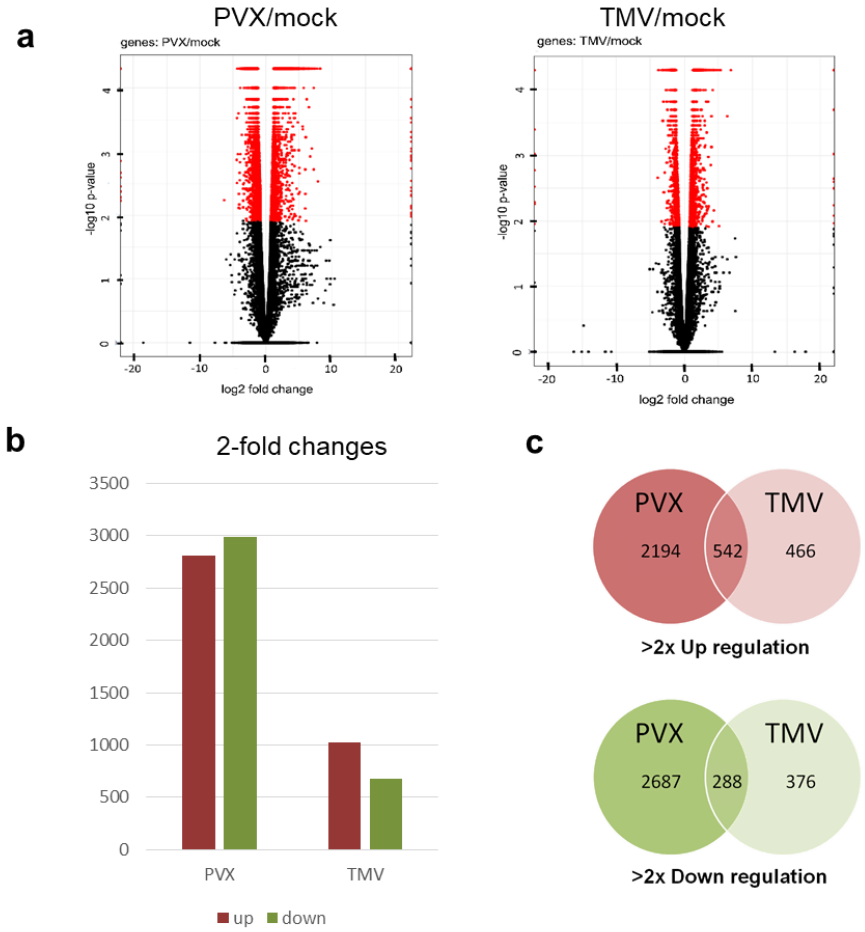


Figure 2. Gene expression changes in virus infected *S. lycopersicum*
To identify differentially expressed genes each virus infected sample was compared to mock treated sample. Obtained fold changes were converted to log2 to generate volcano plots. More than 2-fold changes and p values less than 0.05 were applied to identify DEGs
a/ Volcano plots displays log2 fold changes and p values. b/ Column diagram of number of DEGs showing 2-fold changes. c/ Venn diagram of DEGs identified in the virus infected samples.

From all DEGs, 5711 were identified in PVX and 1672 in TMV infected samples. Among them 2736 and 1008 DEGs were up- and 2975 and 664 DEGs were down-regulated in PVX and TMV infected plants respectively (Figure 2/b). Venn diagrams show that 542 and 288 genes were up- and down-regulated in both infection, but the majority of DEGs were present in PVX infected plants (Figure 2/c). These results suggest that similarly to CymRSV and crTMV infected *N. benthamiana*, PVX cause an acute infection on *S. lycopersicum*, while TMV on this host cause persistent infection resembling to TCV infection on *N. benthamiana*.

2.5 Functional distribution of DEPs/DEGs in virus infected N. benthamiana *and* S. lycopersicum

Comparison of Volcano plots shows that the number of down-regulated genes were much higher in *N. benthamiana* than the number of up-regulated genes, while in *S. lycopersicum* this ratio was about 1:1 (Figure 1 and 2). Probes on the *N. benthamiana* chip could not be directly correlated to changes in the whole genome, some transcripts could be overrepresented, while there are no probes for other ones. Imbalance of the genes represented on the chip could lead to imbalance in the identified DEGs. Irrespectively of this situation function of DEGs (according to their Bin codes) showed that the most differentially regulated genes were ones which participate in protein metabolism in both hosts, followed by DEGs of RNA regulation, signalling and transport processes

(SFigure 5). While in these processes we could find DEGs both up- and down-regulated, DEGs playing role in photosynthesis were exclusively down-, while in stress responses were usually up-regulated. DEGs from these two pathways (photosynthesis and stress) could almost exclusively be found during acute (CymRSV, crTMV and PVX) infections and lack in persistent (TCV and TMV) infections (SFigure 6 and STables 3-6). Investigating the top 20 down- and up-regulated genes in both hosts we have found the same situation: changes were more severe during acute infection (STable 7-10). The most down-regulated genes in *N. benthamiana* were almost the same during CymRSV and crTMV infections and expression of these probes were unaltered during TCV infection, while the most down-regulated genes in TCV infected plants were different and changed with lower magnitude. Unfortunately, most of these probes are not annotated, but those which have known function play role in abiotic stress and photosynthesis (STable 7). The most up-regulated genes in virus infected *N. benthamiana* were stress-related genes, and were very similar in CymRSV and crTMV and different in TCV infected tobacco (STable 8). Most down-regulated DEGs in *S. lycopersicum* have diverse functions, but there are three cytochrome P450s, whose levels were decreased in both PVX and TMV infections. Although some of the top 20 down-regulated genes were similar, the magnitude of their down-regulation was more severe in PVX infected tomato (STable 9). Pathogenesis related proteins (PRs), and different transcription factors, playing role in hormone metabolism were among the most up-regulated genes in virus infected tomato. Although during TMV infection these changes were high, in PVX infected plant changes were even higher (STable 10), supporting our theory that PVX infection of *S. lycopersicum* causes an acute, while TMV a persistent infection on this host.

2.6 Similarities and differences in gene-expression changes during acute and persistent infection according to the microarray and RNAseq analysis

During our previous work we have shown that in systemic leaves of CymRSV infected *N. benthamiana* the level of GAPDH, tubulin, Rubisco, CP29 and histone (H1E) showed drastic down-regulation, the elongation factor (EF) 2 responded with slight decrease, while the level of glutathione S-transferase gene (GST) and heat shock protein 90 gene (HSP90) were increased [18]. Keeping this in mind we have checked the level of these genes in the obtained microarray and RNAseq data. We have found several Rubisco, GAPDH and CPs (chlorophyll binding proteins) specific probes on the microarray chip. Their expression showed severe changes only in the CymRSV and crTMV, and did not alter in TCV infected plants (Figure 3/a upper panel).

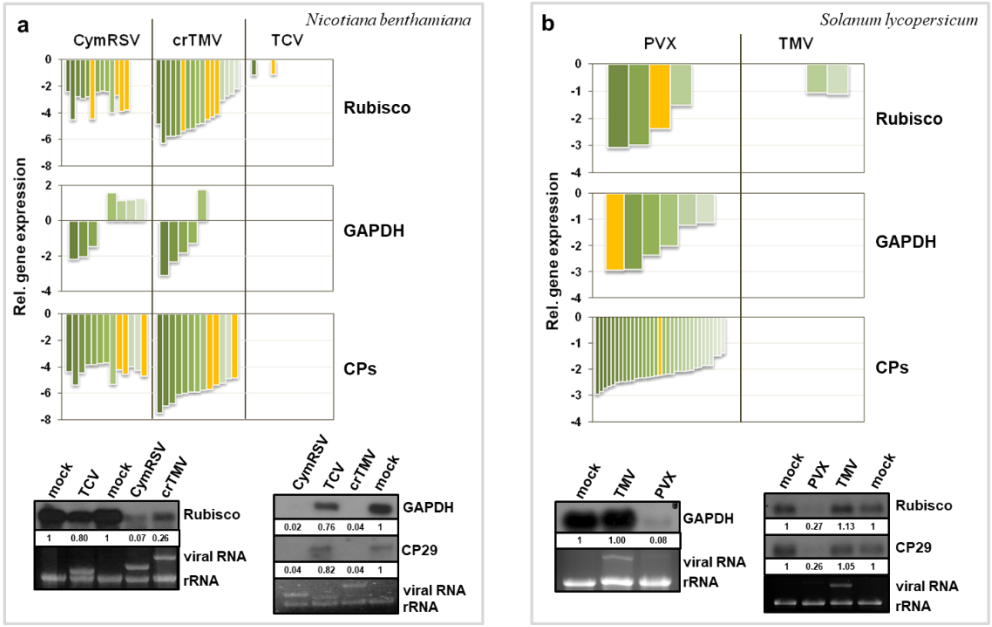


Figure 3. Investigation of gene expression changes of Rubisco, GAPDH and different CPs in virus infected a/ *N. benthamiana* and b/ *S. lycopersicum*. Column diagram in the upper panel shows

log2 fold changes of probes or genes specific for the investigated gene resulted from microarray analysis (*N. benthamiana*) or by RNAseq (*S. lycopersicum*). Lower panel shows Northern blot hybridization made by radioactively labelled probe specific for endogenous genes. Yellow colour on the column diagram shows column which represent value specific for the gene which level was checked by Northern blot hybridization. There was no probe specific for the investigated *N. benthamiana* GAPDH on the microarray chip.

We have cloned fragments of Rubisco, GAPDH and CP29 from both hosts (primers are listed in STables 11, 12) and validated their expression pattern by Northern blot. Results of the Northern experiments correlated very well with the microarray results (Figure 3/a lower panel). RNAseq results of *S. lycopersicum* showed that Rubisco, GAPDH and CPs level decreased during PVX and were not, or only slightly altered in TMV infected plants (Figure 3/b upper panel). Validation of their level by Northern blot showed the same trend (Figure 3/b lower panel). According to the microarray analysis in *N. benthamiana* and RNAseq analysis in *S. lycopersicum* gene-expression of tubulin, EFs and histons were down-regulated during acute virus infections (CymRSV, crTMV and PVX), while they were not, or only slightly altered in persistent virus infection (TCV and TMV) (SFigure 7/a and b). Quantitative RT-PCR results of EF and histone in PVX and TMV infected *S. lycopersicum* validated these findings, showing down-regulation only in PVX infected tomato (SFigure 7/c). Glutathione S transferases are typical stress genes, whose levels were induced during virus infection and this was what we have found in CymRSV infected *N. benthamiana* previously [18]. Expression of GST specific probes in microarray experiments showed up-regulation in CymRSV and crTMV infected plants, while remained unaltered during TCV infection (Figure 4/a upper panel).

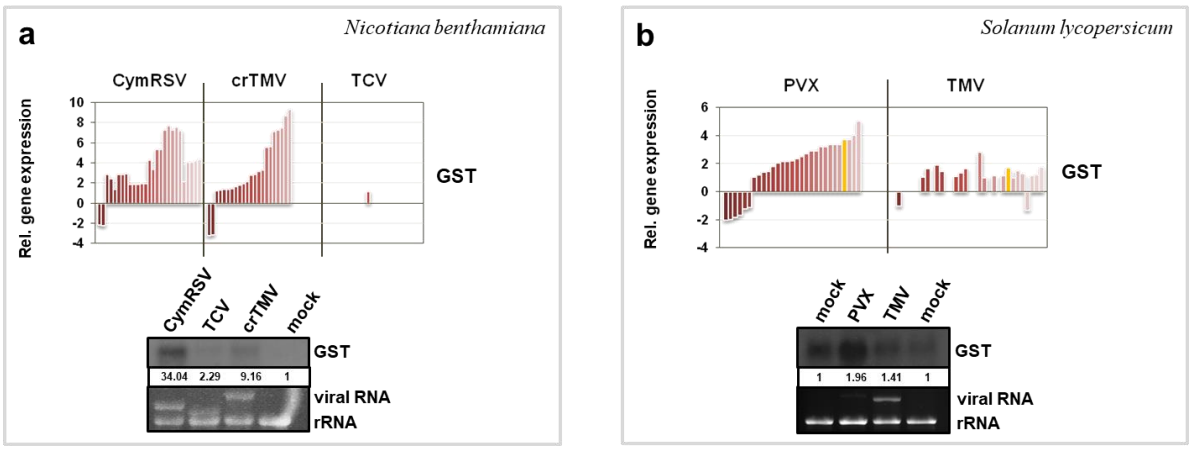


Figure 4. Investigation of gene expression changes of GST in virus infected a/ *N. benthamiana* and b/ *S. lycopersicum*. Column diagram, upper panel shows log2 fold changes of probes or genes specific for GST resulted from microarray analysis (*N. benthamiana*) or by RNAseq (*S. lycopersicum*). Lower panel shows Northern blot hybridization made by radioactively labelled GST specific probe. Yellow colour on the column diagram shows column which represent value specific for the same GST which level was checked by Northern blot hybridization. There was no probe specific for the investigated *N. benthamiana* GST on the microarray chip.

According to the RNAseq results GST genes of *S. lycopersicum* were usually up-regulated during virus infection and these changes were more severe in PVX infected plants (Figure 4/b upper panel). This trend of changes was validated on both hosts by Northern blot analysis using host specific, cloned GST as a template for radioactive probe preparation (Figure 4/a, b lower panels). Beside GST, pathogen related proteins (PRs), systemic acquired resistance genes (SARs) and heat shock proteins (HSPs) are well known stress genes. As we have found no stress gene induction in persistent virus infections (SFigure 6/b) we checked their relative expression in our microarray and RNAseq experiments (SFigure 8). PRs, SARs and HSPs were up-regulated only in CymRSV and crTMV

infected tobacco and PVX infected tomato and their induction were absent or lower in TCV or TMV infected hosts (SFigure 8/a, c). Induction of PR-Q, PR1 and SAR in *N. benthamiana* could be validated by Northern blot, while induction of HSP20 in *S. lycopersicum* was validated by qRT-PCR (SFigure 8/a, b lower panel and c). qRT-PCR results from gene-expression analysis of PR1 and SAR in *S. lycopersicum* showed the opposite trend to the RNAseq results, which we cannot explain, however even in this situation the magnitude of change was much higher in PVX than in TMV infected host (SFigure 8/c). Protodermal factor 1 (PDF1) and lactate dehydrogenase (LDH) were among the DEPs whose levels were severely changed in acute infection in *N. benthamiana*, but stayed unchanged during persistent infection (Figure 5/a, b right panel).

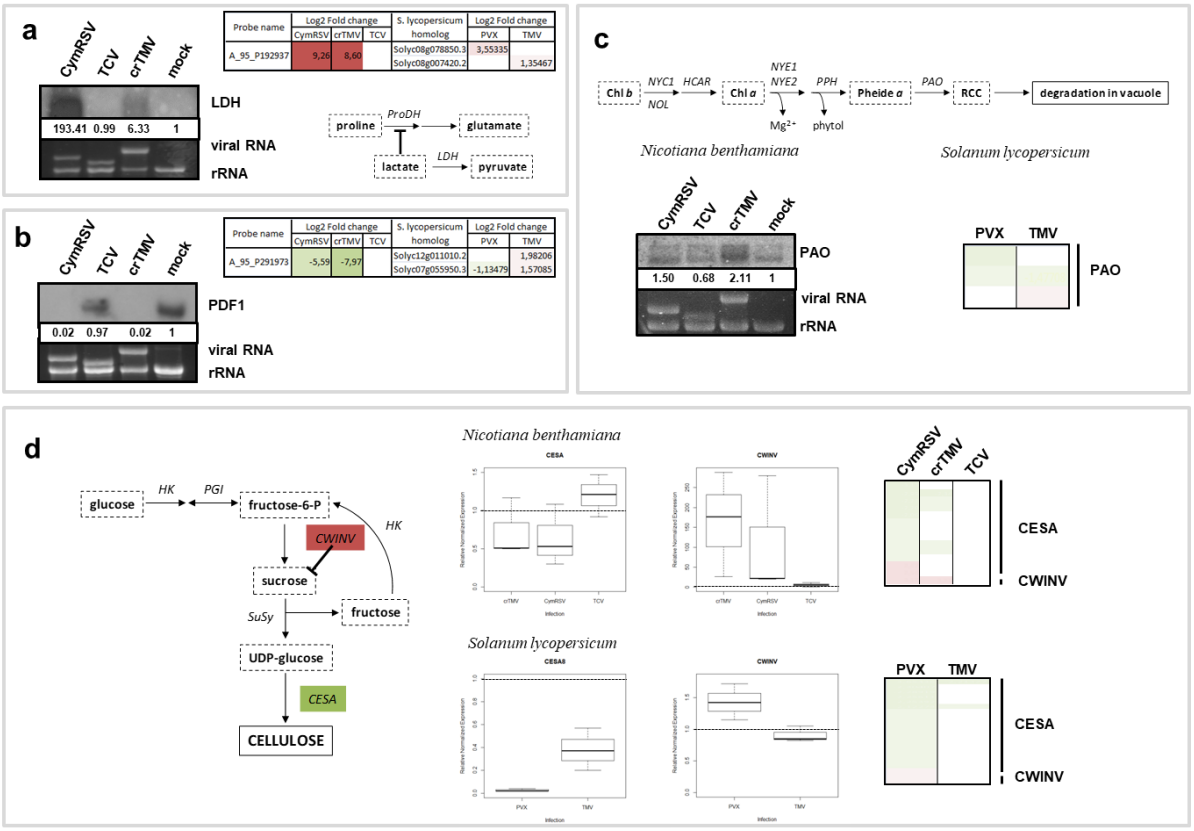


Figure 5. Investigation of gene expression changes of enzymatic pathways in virus infected plants. Gene-expression changes of a/lactate dehydrogenase, b/ protodermal factor1, c/ genes playing role in chlorophyll degradation and in d/cell wall metabolism. Schematic diagrams show role, tables and heat maps shows gene expression changes of the investigated gene, where red show up, while green shows down-regulation. Northern blot hybridizations were made by radioactively labelled gene specific probe. Whisker diagrams on d shows deltadeltaCt results of qRT-PCR from 3 biological and technical replicates using ubiquitin as an internal control.

LDH was shown to play role in proline metabolism [22]. During stress condition fast degradation of proline happens as a result of activation of proline dehydrogenase (ProDH) [23]. While lactate is a substrate of LDH, it is also a competitive inhibitor of ProDH (Figure 5/a lower panel at right). Increase in LDH expression during virus infection enhance ProDH activity allowing fast reaction to stress conditions. PDF1 encodes a putative extracellular proline-rich protein exclusively expressed in the L1 layer of meristems and the protoderm of organ primordia. Its level was strongly down-regulated in acute infection compared to mock and TCV infected leaves (Figure 5/b right panel). Neither PDF1 nor LDL was among the severely changed genes in *S. lycopersicum*. PDF1 was up-regulated during PVX and slightly also during TMV infection. LDH was down-regulated during

PVX infection and slightly up-regulated during TMV infection. Microarray results for PDF1 and LDH could be validated in *N. benthamiana* by Northern blot (Figure 5/a, b left panels).

2.7 Gene-expression analysis behind visible symptom development

Symptom development (chlorosis and yellowing) and leaf senescence in virus infected plants reflects the summary of different molecular and physiological changes [24]. Severe symptom development usually coincides with changes in the photosynthetic activity and also in molecular structure of the host chloroplast (reviewed [25]), but insights of the molecular changes are still elusive. In acute infection down-regulation of photosynthetic processes correlated very well with the severity of observed symptoms (SFigure 6/a). This is in line with the gene-expression changes detected by high-throughput methods in tomato chlorosis virus (ToCV) and tomato yellow leaf curl virus (TYLCV) infected tomato [26], African cassava mosaic virus infected cassava [27], rice black-streaked dwarf and rice grassy stunt virus infected rice [28,29], tomato zonate spot orthotospovirus infected tobacco [30] and three fruit tree virus infected *N. benthamiana* [31]. Chlorosis and yellowing can be a result of chlorophyll (Chl) degradation, an early mark of leaf senescence. The key regulator of Chl degradation is pheophorbide a oxigenase (PAO), catalysing cleavage of the porphyring ring of pheophorbide, resulting in a red Chl catabolite intermediate (RCC), which later degrades in the vacuole (Figure 5/c upper panel). The level of PAO increased during biotic stresses (see for review [32]) and as well as during phytophtora infection in pepper [33]. On the microarray chip we haven't found specific probe for *N. benthamiana* PAO (STable 13), but we have shown by Northern blot that its level was induced during acute infection (Figure 5/c lower panel, left). In contrast to this observation our RNAseq results in *S. lycopersicum* showed three different PAO like DEGs whose expression was decreased in PVX and slightly increased in TMV infection (Figure 5/c lower panel, right, STable 14). During acute-type infection, beside chlorosis and yellowing, slight stunting of both hosts could be observed. If cell wall synthesis is impaired leaf size decrease, or malformation of leaf shape can happen and total growth of the plant is reduced, what could be directly detected in cell wall synthase (CESA) mutant or silenced plants [34]. In contrast, cell wall invertases (CWINV), which convert sucrose into fructose, are usually induced early during any defence [35] (Figure 5/d left panel). In TYLCV infected *S. lycopersicum*, especially when coinfected with ToCV, stunting was directly correlated to CESA8 down- and CWINV2 up-regulation [26]. Investigation of DEPs in *N. benthamiana* and DEGs in *S. lycopersicum* playing role in cell wall metabolism showed the same trend (Figure 5/d right panel, STables 13, 14), i.e. the levels of CESA8 type synthases were down-, while the invertases were up-regulated. These changes were more severe during acute infection in both hosts and were validated by qRT-PCR (Figure 5/d middle panel, STable 14), which could explain the stunted phenotype of these plants.

Different morphological abnormalities which usually present in acute infection can be the result of changes in hormone metabolism. In our experiments DEGS in hormone metabolism pathway were not severely altered (SFigure 5), but slight changes in key regulators, like transcription factors, can have profound effect. Auxins, cytokinins, gibberellic acid and brassinosteroids regulating plant growth, development and elongation can have ambiguous effects during virus infection (reviewed by [36]. Although improper annotation did not allow us to map DEPs and DEGs during these pathways it is obvious that regulation of these pathways were present in acute infection while were almost lack in persistent infection in both hosts (STables 15, 16). Transcription factors regulated by other hormones: ethylene (Et), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) can differentially regulate senescence associated genes (SAGs), which finally lead to chlorophyll breakdown and leaf senescence [37,38]. Changes in the gene-expression pattern of enzymes playing role in hormone biosynthesis were bigger during acute infection suggesting that these pathways are differently regulated (SFigure 9 upper block, STables 17, 18). Altered hormone level leads to differential regulation of hormone response factors and transcription factors: ethylene or ABA response factors (ERFs and ABFs), NAC like transcription factors regulated by ABA or JA (ANACs), MYC transcription factors regulated by JA, JA regulated transcription factors with basic helix-loop-helix motifs (bHLHs) and WRKY transcription factors, especially WRKY6 and WRKY70, having

central role in SA pathway (SFigure 9 middle block, STables 17, 18). Different expression of key regulators cause changes in the expression pattern of enzymes regulated by them: ETHYLENE INSENSITIVE 3 (EIN3), NAC-like transcription factor ANAC029 (ORE1), protein phosphatase 2cs (PP2C), NONEXPRESSOR OF PATHOGENESIS-REGULATED PROTEIN 1 (NPR1), PR1, different senescence-associated genes and enzymes on the PAO pathway [32](SFigure 9 lower block, STable 17, 18). This overall (or combined) effect will finally lead to development of leaf senescence. Changes at each level were more pronounced in acute infections, which explain significant differences between the observed symptoms.

2.8 Physiological changes in virus infected N. benthamiana *and* S. lycopersicum

Host gene down-regulation in acute infections affected several genes, which have important function in the photosynthetic process. To correlate gene-expression changes with physiological behaviour of the infected plants we characterized the photosynthetic response of the host in the above host-virus interactions by the application of various imaging techniques (chlorophyll fluorescence, thermal imaging). Temperature and chlorophyll fluorescence parameters of acute and persistent virus infections differed significantly (Figure 6 and 7).

Evaporative cooling of healthy and infected plants could be distinguished by using thermal imaging approaches. With better stomatal regulation, leaves of healthy plants transpire more water when compared to infected plants. This can be the effect of stomatal closure induced by the pathogen. Thermal images of acute and persistent virus infections were quantified by thresholding evaporative cooled area relative to the temperature of the surrounding air (Figure 6).

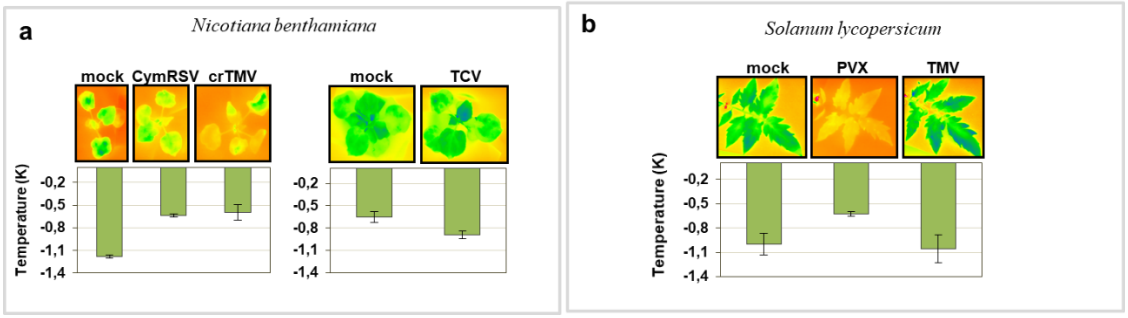


Figure 6. Temperature changes in virus infected *N. benthamiana* and *S. lycopersicum* Thermographic images show the difference between the ambient temperature and the leaf temperature of the virus infected and control plant as a colour image and as a column diagram (in kelvin, K) for a/ *N. benthamiana* infected with CymRSV, crTMV (4dpi) and TCV (8dpi) and b/ *S. lycopersicum* infected by PVX and TMV (13dpi).

We could observe significant transpiration rate in TCV and TMV infected *N. benthamiana* and *S. lycopersicum* respectively. In contrast, in CymRSV, crTMV and PVX infected plants the leaf temperature was found to be higher and similar to ambient air, suggesting that these viruses have drastic impact on the evapotranspiration of plants.

Chlorophyll fluorescence fast kinetics approach also proved to be very effective in predicting the physiological responses. Chlorophyll fluorescence parameters depicting the electron transfer in both acceptor and donor side of photosystem II were drastically effected by CymRSV and crTMV infected *N. benthamiana* plants (Figure 7/a).

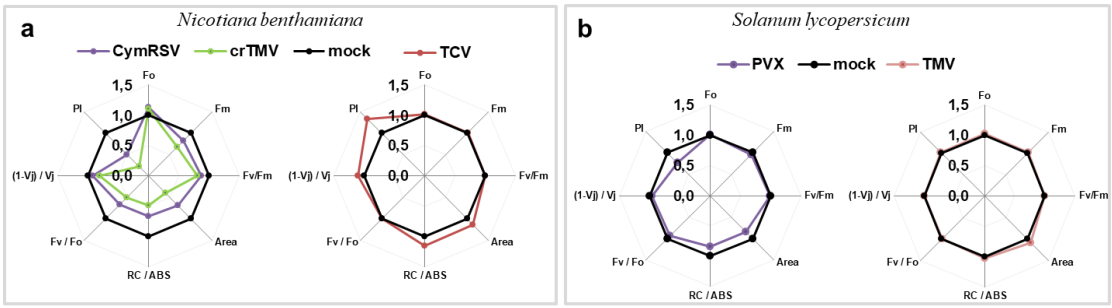


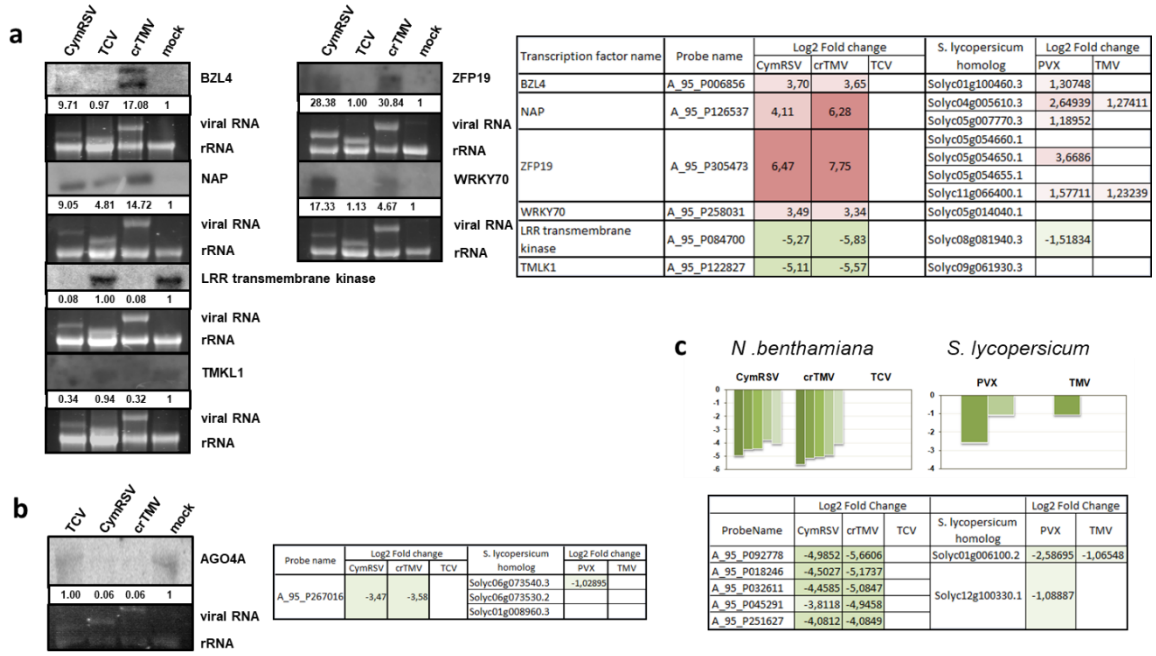
Figure 7. Chlorophyll fluorescence in virus infected *N. benthamiana* and *S. lycopersicum* Spider graphs show calculated chlorophyll fluorescence parameters on a/ *N. benthamiana* infected with CymRSV, crTMV (7dpi) and TCV (11dpi) and b/ *S. lycopersicum* infected with PVX and TMV (8dpi). Parameters shown the values of initial (Fo) and maximal (Fm) fluorescence levels, the Fv/Fm and Fv/Fo (maximal PSII quantum yield) ratios, the area parameter, the (RC/ABS) that is dissipated energy flux per active reaction centre (RC/ABS), the (1-Vj)/ Vj parameter, where Vj = (F2ms – Fo)/Fv, the performance index (PI) measured on third/fourth young fully developed branches from apical tip. The data are shown for the virus infected plants (colour symbols) after normalization to respective values obtained in the mock inoculated plants (black symbols). Data are Means ±SE of 3 independent biological plant replicates per treatment.

In PVX infected *S. lycopersicum* the performance index parameter, the maximal quantum yield for primary photochemistry and the quantum yield for electron transport [39,40] was significantly decreased (Figure 7/b). While in TCV infected *N. benthamiana* and TMV infected *S. lycopersicum* the chlorophyll fluorescence parameters were the same, or a little higher as in the control.

These findings show that in persistent infection the gene-expression changes caused by these viruses did not alter the physiology of the leaves, while in acute infection more severe changes were observed even at a very early point of the infection.

2.10 Investigation of gene-expression changes of possible regulators, which behave variously during acute- and persistent virus infection

In *N. benthamiana* and *S. lycopersicum* we have found similar molecular and physiological changes as a consequence of acute virus infection. These severe changes were absent or slighter during persistent infection in both hosts, suggesting the presence some universal regulatory pathways, which differentiate between viruses belonging to either classes. During our previous work using in vitro transcription assay we showed that shut-off happens at the transcription level, in the nucleus [18] where transcription factors regulate their targets. In virus infected *N. benthamiana* among the most affected genes we have found several transcription factors. We could successfully confirm drastic changes of 6 differently expressed regulatory enzymes by Northern blot in *N. benthamiana*. The transcription levels of basic leucine zipper (BZL)-4, NAP (nucleosome assembly protein) like, zinc finger (ZF) P11 and WRKY70 transcription factors were seriously induced, while the level of a leucin rich repeat (LRR) transmembrane kinase and a transmembrane kinase like (TMKL)-1 were reduced during acute infection (Figure 8). BZL-4 is a member of the BZIP transcription factor family, without any report on its role in biotic stresses. However, its overexpression in tobacco resulted in stunted vegetative growth, shortened internodes, smaller flowers and reduced amount pollen [41]. Moreover, ectopic overexpression of its possible orthologues from Antirrhinum majus repressed transcription of chlorophyll a/b (CAB) binding protein [42]. In line with this, plants with increased BZL-4 level: CmRSV and crTMV infected tobacco and PVX infected tomato, showed reduced growth and the level of CABs were seriously down-regulated, suggesting that this BZL transcription factor is regulated during biotic stress (Figure 8).



Transcription factor name	Probe name	Log2 Fold change			S. lycopersicum homolog	Log2 Fold change	
		CymRSV	crTMV	TCV		PVX	TMV
BZL4	A_95_P006856	3,70	3,65		Solyc01g100460.3	1,30748	
NAP	A_95_P126537	4,11	6,28		Solyc04g005610.3	2,64939	1,27411
					Solyc05g007770.3	1,18952	
ZFP19	A_95_P305473	6,47	7,75		Solyc05g054660.1		
					Solyc05g054650.1	3,6686	
					Solyc05g054655.1		
					Solyc11g066400.1	1,57711	1,23239
WRKY70	A_95_P258031	3,49	3,34		Solyc05g014040.1		
LRR transmembrane kinase	A_95_P084700	-5,27	-5,83		Solyc08g081940.3	-1,51834	
TMLK1	A_95_P122827	-5,11	-5,57		Solyc09g061930.3		

Probe name	Log2 Fold change			S. lycopersicum homolog	Log2 Fold change	
	CymRSV	crTMV	TCV		PVX	TMV
A_95_P267016	-3,47	-3,58		Solyc06g073540.3	-1,02895	
				Solyc06g073530.2		
				Solyc01g008960.3		

ProbeName	Log2 Fold Change			S. lycopersicum homolog	Log2 Fold Change	
	CymRSV	crTMV	TCV		PVX	TMV
A_95_P092778	-4,9852	-5,6606		Solyc01g006100.2	-2,58695	-1,06548
A_95_P018246	-4,5027	-5,1737		Solyc12g100330.1	-1,08887	
A_95_P032611	-4,4585	-5,0847				
A_95_P045291	-3,8118	-4,9458				
A_95_P251627	-4,0812	-4,0849				

Figure 8. Investigation of gene expression changes of regulator factors in virus infected *N. benthamiana* and *S. lycopersicum*. Gene expression changes of a/ regulator genes, b/ Argonaute 4 by Northern blot hybridization made by radioactively labelled probe specific for endogenous genes. Panels in the right show log2 fold changes of probes or genes specific for the investigated gene resulted from microarray analysis (*N. benthamiana*) or by RNAseq (*S. lycopersicum*). c/ Gene expression changes as log2 fold changes of probes or genes specific for of methyltransferases are shown on c, in the upper panel as a column diagram, while in the lower panel as a table.

NAC like transcription factors are well studied and showed to play important role in leaf senescence, being regulated by ethylene insensitive (EIN)-2 pathway [43]. This NAP-like probe on the microarray chip was annotated as a homologue of AtNAP (ANAC019-like) transcription factor, which expresses in floral primordia and up-regulated by APETALA 3 (AP3) and PISTILLATA (PI) [44]. Overexpression of AtNAP resulted in premature senescence [45] and its elevated level was connected to early senescence events [46]. Expression of its *N. benthamiana* and *S. lycopersicum* homologue was induced in acute infections showing that in these infections early senescence has been started (Figure 8). ZF transcription factors play diverse role during stress condition, even during biotic stresses. AtZAP11 expression was induced to response to flagellin22 elicitor peptide during biotic stress in *A. thaliana* [47], while the level of PtiZFP1, a ZF11 homologue in poplar, and was induced during SA and JA treatment and during rust infection [48]. In our experiments we detected increase of ZF11 level in acute infection in both hosts (Figure 8), suggesting that regulatory pathway containing this transcription factors is involved during acute virus infection. WRKY transcription factors are involved in regulation of plant defence responses. WRKY70 itself has been shown to be induced by SA and repressed by JA, interacts with NPR1 and through its activity repress defence connected JA responsive genes during biotic stresses [49] (SFigure 9). According to the latest results it is also involved in brassinosteroid-regulated plant growth [50]. Level of WRKY70 was induced during CymRSV, crTMV infection in *N. benthamiana* and during PVX infection in *S. lycopersicum*, suggesting its important role not only during bacterial, and fungal, but also during virus infection (Figure 8). LRR transmembrane kinase was annotated as a probable inactive kinase, but its *A. thaliana* homologue (At4g23740) was identified to be activated (phosphorylated) during flagellin and xylanase treatment mimicking bacterial and fungal infection [51]. Expression of this LRR transmembrane kinase was decreased during CymRSV and crTMV infection in tobacco and in PVX infected tomato suggesting that it is active and can play a role in early signalling in virus infection

(Figure 8). Another kinase TMKL1 was also identified in our microarray experiments to be down-regulated only during acute infection (Figure 8). This DEP represents a *N. benthamiana* homologue of an Arabidopsis transmembrane kinase, which has unusual kinase-like domain (Valon et al. 1993), but without any know function. Expression of its tomato homologue showed no change during virus infection, so its clear role during virus infection is still elusive (Figure 8).

In the nucleus of the virus infected plants where transcription factors regulate their targets, they themselves are often under the control of RNAi based regulation executed by micro (mi)RNAs and small interfering (si)RNAs (reviewed by [52,53]). 21-22nt long smallRNAs bound into Argonaute (AGO) 1/2/7 regulate their targets by cleavage or translational repression. These post-transcriptional silencing (PTGS) pathways (miRNA, trans-acting siRNA, phased siRNA) were shown to be induced during virus infection [54]. Moreover, transcriptional gene silencing (TGS), in which 24nt long smallRNAs bind into AGO4 can direct dynamic changes in DNA methylation and histone modification, can also be modulated during pathogen attack [55]. In our study we have found down-regulation of both AGO4 and methyltransferases in acute infections in both hosts. In addition, decreased expression of AGO4 could be validated by Northern blot in *N. benthamiana* (Figure 8/b, c) suggesting that this pathway can also be involved in acute infection.

3. Materials and Methods

4. Conclusions

Two different high-throughput methods: microarray hybridization and RNA sequencing were proved to be able to detect changes in the gene-expression pattern of the host induced by compatible virus infection. Changes in the expression level were higher in the DEPs compared to DEGs, which could be a result of different detection methods. Ratio of down-regulated genes in microarray analysis were higher compared to up-regulated ones which could be a result of the presence of overrepresented probes. This trend is a warning signal that interpretation of the most changed gene ontology categories cannot be precisely defined according to the microarray results.

In our work we investigated gene-expression changes during persistent and acute infection on two different hosts and found profound differences. Expression of several genes were changed in acute infections similarly while in persistent infection gene-expression of the host was only slightly altered. Induction of stress genes were also detected, and validated in acute infection, but were missing during persistent infection. Our finding that key regulators of RNAi were altered only in acute infection imply that broad-range host gene down-regulation could be reached using this pathway while without altering this pathway the metabolism of the host could be leaved untouched. Investigation of methylation changes and small RNA pattern of the host in these type of infection in the future could answer this question.

Severe down-regulation of genes playing role in photosynthetic processes has an effect on plant physiology. These effects could be measured by thermal imaging and variable chlorophyll fluorescence transients to follow leaf temperature and photosynthetic activity, respectively. Important agronomical yield losses are usually caused by damage due to acute infection of crops. According to our results this type of infection can be differentiated from latent, persistent infection even before visible symptoms appear. Remote sensing methods are able to monitor these changes [65-67] however, they are based on the knowledge of detailed characterization of both gene expression and the resulting physiological changes in virus-infected plants. Consequently this knowledge could be utilized in the future by remote sensing techniques in precision agriculture based crop management for predicting possible infection risks.

High-throughput sequencing methods lead to the discovery of more and more viruses each day, but it is very difficult to determine the actual importance of these newly discovered viruses. Testing gene-expression pattern of some typical host genes could help to decide if they really are threats.

Coexistence of a virus and its host as a latent virus infection without visible symptoms could be beneficial for both the virus and the host at an evolutional scale. Although we revealed deep insight into the gene-expression regulation during different-type virus infection, the main question: how

viruses can avoid the host reaction during their persistent invasion and reach peaceful coexistence with their host remained unanswered.

Supplementary Materials: The following are available online: SFigures 1_9 Pesti et al 2018 IJMS contains Figure S1-S9, and STables 1_2, Stable 3_6, Stable 7_12 and_Stable 13_19 Pesti et al 2018 IJMS contains Tables S1-S2, S3-S6, S7-12 and S13-19 respectively.

Author Contributions: conceptualization, Z.H. and É.V.; methodology, I.V. and É.V.; validation, R.P.; formal analysis, R.P., L.K., K.P., É.V.; investigation, R.P., L.K. and K.P.; writing—original draft preparation, É.V.; writing—review and editing, R.p., L.K., K.P., I.V., Z.H. and É.V.; visualization, R.P., L.K., K.P. and É.V.

Funding: This research was funded by National Research, Development and Innovation Office – NKFIH: PD78049, K108718 and K119783; EU EPPN2020 (Grant agreement no.: 731013), Pesti Réka is a PhD student of the Doctoral Programme of Biological Sciences at Szent István University.

Acknowledgments: We would thank Katalin Éder for microarray experiments and for Ferenc Marincs for the initial statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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