



SZENT ISTVÁN UNIVERSITY

**GENE EXPRESSION CHANGES DURING THE USAGE OF
VIGS VECTORS AND EXAMINATION OF THE SMALL RNA
BINDING ABILITY OF A VIRAL SUPPRESSOR PROTEIN**

Ph.D. thesis

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Gödöllő

2017

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Introduction

RNA interference (RNAi) is an important defence mechanism against viruses, transgenes, and other molecular parasites. The RNA interference pathway is induced by double stranded RNAs (dsRNAs) resulting from virus replication. Viruses induce a defence response in their hosts involving recognition by the Dicer enzyme, which cleaves long double-stranded RNA (dsRNA) molecules into short, 20-25 nt double stranded small interfering (siRNAs). One strand of the siRNA is incorporated into the ARGONAUTE (AGO) protein which is the central component of the RNA-induced silencing complex (RISC), this complex cleaves or translationally inhibits all RNAs having sequence complementarity to the incorporated siRNA. RNAi plays an important role not only in viral infections but also in regulating endogenous genes such as micro RNAs (miRNAs). miRNAs control mRNAs of endogenous genes by cleavage or translation inhibition and can have important role also in viral infections.

There is an arms race between plants and viruses, therefore viruses have evolved strategies to successfully survive, for example, they have developed RNA silencing suppressor proteins. Viral suppressor proteins, can inhibit the RNA silencing process at several points. The p19 protein of Tombusviruses is capable of binding siRNA or inducing miR168, which plays an important role in controlling the AGO protein of the central RISC complex.

RNA interference-based methods are suitable for determining the function of endogenous genes because of the specificity and efficiency of this mechanism. Over the last decades, plant viral vectors became commonly used to determine gene functions. Virus induced gene silencing (VIGS) is one of them and used as a reverse genetic approach to determine function of gene of interest by downregulating its gene expression level. It is a commonly used method for gene function studies, because it has many advantages such as: easy-to-use, fast, provides the generation of a loss of function phenotype and the cost of VIGS experiment relatively low.

VIGS uses viral vectors carrying a fragment of the gene of interest. Upon infection, the modified virus (virus vector) induces gene silencing not only against viral sequences but also for the inserted plant gene. During viral replication long double-stranded RNA are formed and induce RNAi. These dsRNAs are recognized and cleaved by the Dicer like enzymes into short, 20-25 nucleotide long short RNAs, siRNAs. One strand of the siRNA is incorporated into RNA-induced silencing complex which cleaves or translationally inhibit activity of all RNAs having sequence complementarity to the incorporated siRNA. This leads to the degradation of the corresponding plant mRNAs and a loss of function phenotype will appear.

On the other hand, usage of virus vectors have some limitations, because in compatible plant-virus interactions, the virus can alter the gene expression pattern of the host. Viruses are

able to induce efficiently host gene mRNA down regulation (*shut-off*) of important housekeeping genes. The *shut-off* (during VIGS vector based experiments) often affects household genes used as reference value in quantitative PCR measurements. Before starting to use a virus vector for VIGS, is essential to test its effect on the expression of plant genes.

Objectives

The primary purpose of our work was to test the previously developed virus vectors in terms of how the VIGS vector as a pathogen of the host can cause unwanted changes of the host plant gene-expression pattern and we wanted to clarify whether is there any relationship between induction of the miR168 level and the small RNA binding ability of the p19 viral suppressor protein.

Our aims were:

1. to investigate the presence of the *shut off* phenomenon in TMV, PVX, TRV virus vector infected tobacco and tomato plants and in BSMV virus vector infected wheat, by monitoring host plant gene expression levels of housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (Gapdh), ribulose-1,5-biphosphate-carboxylase oxygenase (Rubisco), actin, elongation factor (Ef), cyclophilin (Cph) and tubulin.
2. to examine the correlation between the small RNA binding and the induction of the miR168 ability of the viral suppressor protein p19.

Methods

PLANT MATERIAL

Nicotiana benthamiana, *Solanum lycopersicum* (Kecskeméti jubileum variety) and *Triticum aestivum* (Bezostája variety) plants were grown in a tempered light room, under a 16 h light / 8 h dark regime at 21–22°C.

Our experiments were carried out in two biological replicates; 2 young leaves of 3-4 plants were pooled and processed for Northern blot analysis.

INFECTION WITH VIRUSES

Tobacco mosaic virus (TMV-VIGS), Potato virus X (PVX-VIGS), and Barley stripe mosaic virus based BSMV-VIGS) and Tobacco rattle virus based (TRV-VIGS) based vectors were used in our experiments. Both empty vectors and vectors carrying a piece of host PDS were linearized: TMV-VIGS with KpnI, PVX-VIGS with SpeI, BSMV α and γ with MluI and BSMV β with SpeI enzyme. *In vitro* transcripts were synthesized from these linearized templates. *In vitro* transcripts were used to inoculate the appropriate host, BSMV α , β , and γ were mixed in a 1:1:1 ratio. TRV1 and TRV2-VIGS constructs were introduced into *Agrobacterium* strain C58C1, mixed in a 1:1 ratio (OD₆₀₀ =1.0) and used for agroinfiltration. The control plants were infiltrated with *Agrobacterium* strain C58C1 containing only TRV1.

We infected *Nicotiana benthamiana* plants with wild-type Carnation Italian ringspot virus (CIRV) or mutant viruses (CIRV + DI, CIRV19Stop and CIRV-3M) by mechanical inoculation using *in vitro* RNA transcripts.

In our experiments, in parallel with the viral infection, healthy plants were subjected to mechanical inoculation, but in the absence of the virus. These plants are called mock plants, which are used as negative controls.

PROTEIN EXPRESSION

To test their small RNA binding activity, CIRV p19 and p19-3M VSRs were expressed and purified as a GST fusion protein in *Escherichia coli* strain BL21 (DE3) at 22°C for 20 h according to the manufacturers' instructions (GE Healthcare Life Sciences). Bacterial pellets were suspended, and the fusion protein was purified from the soluble fraction of the lysate by batch method incubating with glutathione agarose resin for 30 min at 21°C. The resin was washed four times with cleavage buffer was incubated with thrombin (Amersham Biosciences) for 3 h at room temperature. After cleavage, the liberated p19 protein was collected. Subsequently, the small RNA binding ability of purified suppressors was evaluated by electrophoretic mobility shift assay.

ELECTROPHORETIC MOBILITY SHIFT ASSAY PROTEIN EXPRESSION

Radioactively labelled synthetic siRNAs were added to purified suppressors. The sequences of the RNA oligonucleotides used were 5' UGAUUAUUGGCACGGCUCAAUC 3' and 3' UUGAGCCGUGCCAAUAUCAUC 5'. Purified p19 and p19-3M protein VSRs (3 nM) were added to 1 pM radiolabelled siRNA in binding buffer. Binding reactions were incubated at 25°C for 30 min, complemented with 3 µl of loading dye. Thereafter the reaction was analyzed by electrophoresis at a constant 50 V for 1.5 hour through a 6% TBE DNA retardation gel in 0.5X TBE. The gels were dried in vacuum, and then exposed.

Results

1. NON-TARGETED EFFECTS OF VIGS VECTORS ON HOST ENDOGENOUS GENE EXPRESSION

In our experiments we investigated, the presence of the *shut-off* phenomenon in plant-virus interaction in which endogenous gene function were established by VIGS vectors.

Nicotiana benthamiana, *Solanum lycopersicum* and *Triticum aestivum* plants were infected with the most commonly used VIGS vectors: TMV, PVX, TRV, and BSMV-based virus vectors. The host plants were inoculated in parallel with VIGS vector that did not contain anything at the cloning site (VIGS-Empty) or containing a piece of endogenous PDS gene of the plant (VIGS-PDS). We investigated the expression level of endogenous genes by Northern blot analysis (Rubisco, Gapdh, tubulin, elongation factor, Cph and actin), the expression changes of these genes well indicate the presence of the *shut-off* phenomenon and they are often used as a reference value in VIGS experiments.

By comparing *N.benthamiana* plants infected with PVX and TMV (Empty, PDS) vectors, to mock plants, we found that expression levels of a number of endogenous genes have decreased, for example, Rubisco, Gapdh and tubulin, so *the shut off* phenomenon is present in these plants . The level of the Cph gene increased in PVX, TMV and TRV-VIGS infected tobacco plants compared to mock-inoculated plants.

In the case of tobacco plants infected with TRV-Empty VIGS vector, the investigated mRNA levels were unchanged in comparison to mock-inoculated plants. In contrast, in tobacco plants infected with TRV-PDS VIGS vector, it was found that Rubisco, Gapdh and actin mRNA levels were significantly decreased compared to the mock-inoculated plants. This observation suggests that the introduced fragment can modify the effect of the infecting virus on the host's gene expression pattern.

In the PVX-Empty VIGS vector infected tomato plants the expression level of Rubisco, Gapdh and tubulin was slightly decreased, whereas in the PVX-PDS VIGS vector infected plants the expression level of Rubisco, Gapdh and tubulin was drastically down regulated compared to mock-inoculated plants.

In the TMV-Empty VIGS and TRV-empty vector infected tomato plants we did not found drastic changes in the expression of the investigated genes. On the other hand, in plants infected with PVX-PDS and TRV-PDS VIGS vector, the expression levels of the tested genes decreased drastically with the exception of Cph.

Similarly to the previous findings in tobacco, in tomato plants, we also observed that, the presence of integrated foreign sequence in the VIGS vector may influence the formation of the *shut-off*.

To test whether this difference is a consequence of the drop in chlorophyll content due to VIGS induced RNAi of PDS mRNAs, we cloned neutral GFP pieces (100bp and 300bp long) into the TRV-VIGS vector and infected GFP transgenic plants. The levels of Rubisco and Gapdh in these infections were decreased just like after TRV-PDS infections, suggesting that the decrease in chlorophyll level and in the expression levels of these genes are independent processes. To find out whether changes in the expression of these genes are dependent on the size of the cloned piece of DNA, we tested changes in the expression of these genes after infection with TRV-Upf1 (582bp). We found the same decreased Rubisco and Gapdh levels, as with different lengths of GFP, suggesting that this phenomenon is independent from the size and function of the cloned piece of DNA.

In BSMV-VIGS (Empty, PDS) vector-infected wheat, we found that the Gapdh, Rubisco, and actin expression levels drastically decreased compared to mock plants.

2. STUDY OF THE CORRELATION BETWEEN THE SMALL RNA BINDING ABILITY OF THE VIRAL SUPPRESSOR PROTEIN P19 AND THE INDUCTION OF THE miR168

We have investigated whether the viral suppressor protein p19 small RNA binding ability and the miR168 induction ability are independent functions or not. To analyse the siRNA binding ability, CIRV p19 and p19-3M VSRs were expressed and purified as a GST fusion protein in *Escherichia coli* strain BL21 (DE3). Subsequently, it was demonstrated by gel mobility shift assay experiment that the mutant suppressor protein lost its siRNA binding ability. We found that while the wild type p19 VSR showed extremely high affinity to labelled siRNAs, p19-3M completely lost its siRNA binding ability showing no residual binding activity.

Nicotiana benthamiana plants were infected with CIRV, CIRV and DI RNA, CIRV19Stop and CIRV-3M (mutant virus carrying p19-3M) and the level of miR168 was examined. In plants with CIRV and DI RNA, and CIRV-3M infection, we encountered miR168 induction. This suggests that the small RNA binding ability of p19 suppressor protein and the induction of miR168 evolved independently.

3. INVESTIGATION OF MIR168 INDUCTION IN VIRUS INFECTED GRAPEVINE

Due to the viral infection in herbaceous plants increased the miR168 levels; we examined whether this phenomenon is present in virus infected woody plants as well. We examined the level of miR168 in infected grapes with Grapevine Syrah virus 1 (GSyV1) Pinot gris virus (GPGV). In GSyV1 and GPGV-infected samples, there was no increased miR168 expression compared to virus-free vine samples.

New scientific results

1. According to our results the optimal VIGS vector for *Nicotiana benthamiana* is TRV-VIGS vector. PVX, TMV-VIGS vectors are causing severe changes in the host gene expression.
2. In *Solanum lycopersicum* host the TMV-based VIGS vector was the most suitable for gene function studies, because the TMV-VIGS vector is not causing significant changes in the host plant's gene expression.
3. We demonstrated that the use of BSMV-VIGS vector in the *Triticum aestivum*, is responsible for various alterations in the host plant's gene expression.
4. We have proved that unrelated sequence elements in the TRV-VIGS vector, drastically alter their effect on the gene expression pattern of the host plants. The gene expression changes depend on the presence of the foreign sequence built into the VIGS vector, but they are independent of its length and function.
5. We proved that the CIRV p19-3M mutant, produced by us, has lost its small RNA binding ability.
6. We found that the presence of DI RNA does not influence the induction of the miR168 mediated by p19 suppressor protein, therefore the small RNA binding ability of the p19 suppressor protein and the induction of miR168 are independent processes.
7. We demonstrated that the expression level of the miR168 gene was not induced in GSyV1 and GPGV infected grains

Conclusions and suggestion

NON-TARGETED EFFECTS OF VIGS VECTORS ON HOST ENDOGENOUS GENE EXPRESSION

In our experiments, we tested expression levels of endogenous genes (Rubisco, Gapdh, Ef, actin, tubulin) with Northern blotting, these endogenous genes are frequently used as internal controls in VIGS experiments with qPCR methods. If we are testing the function of certain genes, it is important that the VIGS vector does not cause expression changes in other genes. In case of experiments made by using VIGS vectors, the reference genes, used as a quantitative reference values for quantitative RT-PCR, should be selected carefully. In order to get trustworthy results in gene expression studies made by qRT-PCR method, it is essential to select a reference gene with stable expression.

Our results showed, that in TMV-VIGS, PVX-VIGS and TRV-VIGS (empty, PDS) infected tobacco and tomato plants or in BSMV-VIGS (empty, PDS) infected wheat plants the expression level of housekeeping genes (Gapdh, Rubisco, Ef, actin, tubulin), used as a control in VIGS studies, were altered, therefore the *shut-off* phenomenon is present in these cases. The expression level of these genes varies and highly depends on the given plant virus interaction, therefore in studies regarding the function of genes, it is advised to select only suitable virus vectors without *shut-off* phenomenon in the given plant for these type of studies. Our results indicate, that in tomato plants the TMV-VIGS vector don't cause significant changes in gene expression, therefore is optimal for gene function studies.

More importantly we found that, the foreign gene sequences built in the VIGS vector (PDS 100vbp GFP, GFP 300 bp, 538 bp UPF) alters the gene expression and affects the formation of *shut-off* phenomenon. The molecular mechanism of this phenomenon is unknown.

In BSMV-VIGS vector infected wheat, we found that the most widely used reference genes (actin, Gapdh, and Rubisco) were drastically down regulated compared to mock plants. When applying the BSMV-VIGS vector, the results must be evaluated carefully, because this type of VIGS vector can drastically alter the gene expression pattern of the host.

It is thought-provoking that in BSMV-VIGS infected wheat, which reference gene, is most suitable for evaluating the results. Some researcher recommends the simultaneous use of four reference genes (Gapdh, 18SRNA, TUBB and EIF4A) in studies made by qRT-PCR.

We investigated various VIGS vectors and we found that, in many cases, they cause severe gene expression changes in the host plant. Our experiments revealed that the presence of a foreign sequence in the VIGS vector can influence the gene expression pattern of the host plant, so if we are using the VIGS vector, it is necessary to be more careful when evaluating the results.

Our results highlight the importance of cautious interpretation of the results obtained and the careful selection of both reference genes and genes whose function is to be tested in VIGS experiments.

STUDY OF THE CORRELATION BETWEEN THE SMALL RNA BINDING ABILITY OF THE VIRAL SUPPRESSOR PROTEIN P19 AND THE INDUCTION OF THE miR168

We wanted to find out if the small RNA binding and miR168 inducing functions of the p19 protein are linked together or not? Our results showed that the small RNA binding property of p19 is not needed for the induction of miR168, therefore these functions evolved independently.

Overall, in the plant viral arm race, the virus needs both ability: the small RNA binding and the induction of miR168 to effectively inhibit the plant's defensive mechanism.

We investigated the effects of GSyV1 and GPGV virus infection in grape plants. In these cases the level of miR168 was not elevated. This could be explained by the fact that in woody plants, in contrast to herbaceous plants, there is no need for fast and reversible regulation of virus infection, because the course of virus infection is longer and the level of the virus is lower.

Publications

Publications in international scientific journals

1. **Enikő Oláh, Réka Pesti, Dénes Taller, Zoltán Havelda and Éva Várallyay. (2016)** Non-targeted effects of VIGS vectors on host endogenous gene expression. Archives of Virology 161, 2387-2393. IF:2,2
2. **Varallyay Eva, Olah Eniko, Havelda Zoltan. (2014)** Independent parallel functions of p19 plant viral suppressor of RNA silencing required for effective suppressor activity NUCLEIC ACIDS RESEARCH 42: pp. 599-608. IF:8,4

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5. **Enikő Oláh, Zoltán Havelda, Éva Várallyay (2013)** Limitation in usage of VIGS vectors in determination of function of endogenous genes in plants, Hungarian Molecular Life Sciences, Siófok, 05-07 May 2013 ISBN: 978-615-5270-02-4
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