Characterization of AGO-binding silencing suppressors and creating the first artificial RNA silencing suppressor

Ph.D. Thesis

Edit Zsuzsanna Szabó

Supervisor: Lóránt Lakatos Ph.D.

Department of Dermatology and Allergology,
University of Szeged
Szeged, Hungary
2015

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1 Introduction

1.1 RNA silencing

RNA silencing is an evolutionary conserved mechanism in many, if not all, eukaryotes to target and degrade aberrant endogenous or exogenous RNA molecules.

RNA silencing can be regarded as a two-step reaction: initiation and effector phases. The *initiation* step is characterized by the endonucleolytic cleavage of longer dsRNA molecules into siRNA or miRNA species. These small RNAs are 21-26 bp in size and contain 2 nt 3'-overhangs and 5'-phosphorylated termini, which are characteristic for their production by RNase-III-type enzymes from the Drosha/Dicer protein family. The siRNA and miRNA molecules differ in their origin and structure. While siRNAs originate from perfect complementary dsRNA duplexes, miRNAs contain mismatches, bulges or G:U wobble base pairs. MiRNA molecules are host-encoded and derive from long non-coding single stranded (ss) RNAs

In the *effector* step of the RNA silencing pathway, one strand of small RNA duplex is loaded onto a ribonucleoprotein complex, the RNA induced silencing complex (RISC) for sequence specific identification of target RNAs. Enzymatic activity of members of the Argonaute (AGO) protein family enables the preassembled RISC to slice complementary mRNAs or arrest their translation. Molecular and genetic analysis in plants and animals have revealed many RNA silencing pathways in which different small RNAs and RNA silencing proteins are key players.

As it acts as antiviral mechanism, the siRNA-mediated pathway is the most relevant RNA silencing pathway for plant virology. Antiviral silencing acts in the cytoplasm and is initiated predominantly by highly-structured viral ssRNAs, dsRNA replication intermediates of plant viruses, cytoplasmically replicating viruses or dsRNA produced by plant RNA dependent RNA polymerase (RDR) action (RDR1 or RDR6) The newly synthesized dsRNAs are subsequently recognised by DCL4 to generate secondary siRNAs of exclusively 21 nt in size, thereby amplifying silencing. Interestingly, a second class of antiviral siRNAs (24-26 nt) can be generated specifically in plants, which seem to be involved in long-distance systemic silencing.

1.2 Argonaute proteins

Argonaute proteins play key roles in diverse RNA silencing pathways. In *A. thaliana* there are 10 Argonaute proteins. The founding member of the AGO gene family was discovered through the identification of *Arabidopsis* mutants that exhibit pleiotropic developmental defects. AGO1 mutants were shown to be deficient in transgene silencing as well as they were hypersensitive to plant viruses. Furthermore, AGO1 is playing a key role in miRNA regulated developmental processes.

1.3 Viral suppressors of RNA silencing

Plant viruses are efficient pathogens, which are able to infect and invade distinct plant species. The fact that most viruses have evolved RNA silencing suppressors underlines the antiviral nature of RNA silencing and reveals a pathogen counter defensive strategy. Viruses often cause severe symptoms, which suggests that the suppressor proteins are able to effectively disturb the RNA silencing pathways of the host. Many RNA silencing suppressors have been identified so far. Inhibition of viral RNA recognition and dicing as a silencing suppressor strategy is known only in a couple of cases among plant viruses.

RISC assembly can be prevented by siRNA sequestration. The p19 proteins of tombusviruses bind 21 nt siRNAs with high affinity. *Tobacco etch virus* HC-Pro, *Tobamovirus* P122 and *Tomato aspermy cucumovirus* 2b protein also compromise RISC assembly by preventing si/miRNA incorporation.

Interestingly, a sole RNA silencing suppressor has been identified so far that has the capacity to inhibit the preassembled RISC complex. The P1 protein of the *Sweet potato mild mottle virus* (SPMMV) binds AGO1 via its WG/GW domains and hampers RISC activity by a yet unidentified mechanism.

However, suppressors can be grouped whether they inhibit RISC assembly or preassembled RISC complexes. Thus, suppressors impairing viral RNA recognition or dicing, sequestering small RNAs or directing AGO degradation, they all inhibit RISC assembly.

Tobacco rattle virus (TRV), the type member of the *Tobravirus* group, has a bipartite genome consisting of single-stranded, positive sense RNAs. The larger

genomic RNA, named RNA1, contains four open reading frames (ORF). The first two ORFs encode a protein with a molecular weight of 134K and a 194K protein, which is produced by read-through translation of the 134K gene. The two products are expressed directly from the viral genomic RNA and have a putative function in viral RNA replication. The third ORF encodes a 29K protein with a role in cell-to-cell movement of the virus. Finally, the 3'-end ORF encodes a small cysteine-rich 16K protein having RNA silencing suppressor function. The 29K and 16K proteins are translated from subgenomic RNAs. The genomic RNA2 contains the coat protein gene and the gene product determining the vector transmissibility.

2 Aims

More than 40 plant viral RNA silencing suppressors have been identified so far, however the exact working mechanism was determined for only a few of them. To better understand the working mechanism of Argonaute binding RNA silencing suppressors, the following aims have been set:

1. Characterization of the 16k silencing suppressor of the *Tobacco rattle virus*

2. Determination the minimal silencing suppressor domain of the *Sweet potato mild mottle virus* P1 protein

3. Restoring the AGO hook of the *Sweet potato feathery mottle virus* P1 protein

3 Materials and methods

3.1 Nicotiana benthamiana propagation and agroinfiltration

Wild-type (wt) and GFP16c/RDR6i *N.benthamiana* plants grown in soil under normal growth conditions were used for, virus infection and agroinfiltration. Plants were grown in Phytotron (Versatile Enviromental Test Chambers; Sanyo, Tokyo Japan) under a 14 h light (50 mE m±2s±1) and 10 h dark regime at 23 ˚C.

Plasmid DNA was introduced into the *Agrobacterium tumefaciens* C58C1 strain by triparental mating. *Agrobacterium* strains harboring the expression plasmids were cultured at 28˚C in LB medium. Cultures were centrifuged then resuspended in the induction solution. OD=0.3 for suppressor and OD=0.1 for reporter genes was used.

3.2 Sample preparation and blot analysis

For Western blot analysis, samples were extracted in 5x loading puffer and boiled for 5 minutes and separated on 10% SDS-PAGE. Anti-HA (1:3000 in 5% dry milk) and anti-GFP (1:5000) was used for HA and GFP tagged samples, respectively. For co-immunoprecipitation anti-HA and anti-myc antibody was used (1:5000).

For RNA isolation, leaf tissue was frozen in liquid nitrogen then extracted in equal volume isolation buffer (200 mM Na-acetate pH=5.2, 1.0 % SDS and 10 mM EDTA pH=8.0) and water-saturated phenol. Organic and non-organic phases were separated by high-speed centrifugation at room temperature for 5 minutes. Then the water phase was extracted with equal volume of phenol-chloroform and phases were separated as described above. RNA was precipitated by adding 2 volumes of 96 % ethanol. RNA was sedimented by high speed centrifugation (10 minutes at 4 ˚C), pellet was washed with 70 % ethanol then dried under vacuum. Finally, RNA was solubilized in sterile water.

For the Northern blot analysis, high molecular weight RNA was separated on 1.0% agarose, 2.2 M formaldehyde and 1×MOPS containing gels. Small RNA was separated on 12 % poly-acrylamide and 8 M urea containing vertical gels in the presence of 0.5×TBE. RNA was transferred to Hybond-N membrane in the presence of 20×SSC by capillary blotting. Membrane was prehybridized in PerfectHyb (Sigma-

Aldrich) for 1 hour at 42 °C. P^{32} labeled DNA probe was denatured by boiling then added to the hybridization reaction and incubated for overnight at 42 °C. Washing the membrane was performed three times in 1×SSC, 0.1% SDS at 65 °C, then membrane was exposed to a storage phosphor screen (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences).

3.3 DNA construction and molecular cloning

We prepared the N-terminal 360, 305, 210 and 120 amino acid containing versions by PCR.

SPFMV P1 was amplified by degenerated primers then mutagenized by site-directed mutagenesis. Histidine residue at position 109 and the tyrosine residue at position 139 were changed to tryptophan. Mutants were checked by DNA sequencing.

3.4 Immunoprecipitation

Extracts for immunoprecipitation were prepared in IP buffer, then incubated for 1 hour at 4 °C with beads containing anti-HA antibody (Roche). Beads were then washed with IP buffer three times. Half of the eluates were used for RNA isolation using 400 µl 2×PK buffer (200mM TRIS pH=7.5, 300 mM NaCl, 20 mM EDTA pH=8.0), which was extracted by equal volume of phenol-chloroform. RNA was precipitated with 2 volume of 96 % ethanol using 10 µg of glycogen. The other half of the eluate was directly used for Western blotting. Commercially available antibodies were used for detecting GFP (Roche), HA-tag (Roche), myc-tag (Sigma).

4 Results

4.1 Characterization of the Argonaute binding 16K protein of the *Tobacco rattle virus*

Tobacco rattle virus (TRV) infection is tightly under the control of the RNA silencing machinery as deduced by elevated TRV levels in plants in which RNA silencing components are genetically inactivated. 16K interferes with cell-to-cell movement and systemic propagation of silencing but not transitive RDR-dependent amplification of RNA silencing.

Using the standard agroinfiltration assay with the 35S-GFP reporter leads to the *de novo* formation RISC complexes. DsRNA and siRNA duplex-binding silencing suppressors impair target cleavage activity by inhibiting RISC assembly. To be able to identify and characterize RNA silencing suppressors that inhibit the activity of programmed RISC (active RISC), recently sensor-based assays were developed.

Plants were infected with the *Cymbidium ringspot virus* (CymRS) mutant Cym19stop, which triggers RNA silencing in upper leaves. These leaves are resistant to challenge inoculation by viruses with sequences homologous to the primary virus, indicating that they contain active RISC complexes. To analyze the activity of CymRSV-activated RISC and suppressor effects on this activity, GFP-Cym sensor RNAs and suppressor proteins were analyzed in Cym19stop-infected *N. benthamiana* plants. SiRNA sensor constructs contained a GFP-encoding ORF fused to a sequence of 194 nt of CymRSV (GFP-Cym) or 204 nt of PoLV (GFP-PoLV). The PoLV sensor construct was used as a negative control, as it does not contain sequence similarity to CymRSV.

Nonsymptomatic leaves were co-infiltrated with *Agrobacterium* strains carrying the GFP-Cym or GFP-PoLV sensor and 16K cDNA constructs. SPMMV P1 was used as a positive, while HC-Pro was used as a negative control.

Consistently, the amount of GFP protein was significantly lower from the GFP-Cym sensor than from the GFP-PoLV sensor. Moreover, GFP-specific RNA shorter than the predicted sensor mRNA transcript was detected in RNA samples recovered from GFP-Cym, but not GFP-PoLV-infiltrated patches co-infiltrated with 16K and HC-

Pro. As expected, SPMMV P1 inhibited viral siRNA loaded preassembled RISC, which was indicated by the increased fluorescence in infiltrated patches and the lack of shorter GFP derived cleavage product on Northern blot.

To examine effect of 16K protein on miRNA-programmed RISC activity, we used the second *in vivo* test system developed. This assay takes advantage of the fact that miR-171 exists in fully developed *N. benthamiana* leaves in active RISC complexes. Thus, lack of inhibitory capacity of an RNA silencing suppressor to active RISC results in degradation of the mRNA transcribed from the sensor and reduced GFP accumulation.

Agrobacterium co-infiltration experiments were carried out using a GFP-171.1 sensor coinfiltrated with 16K of TRV. SPMMV P1 was used as a positive, while HC-Pro was used as a negative control. GFP fluorescence and protein accumulation was reduced in patches expressing the GFP-171.1 sensor compared to patches expressing the GFP-171.1+SPMMV P1 constructs. In the case of GFP-171.1+16K infiltrated patches, either GFP fluorescence or GFP protein level were much lower than in GFP-171.1+SPMMV P1 patches indicating that 16K, similarly to TEV HC-Pro, is not able to inhibit preassembled RISC complexes. Similarly, accumulation of GFP-171.1 sensor transcripts were reduced in empty vector, 16K and HC-Pro infiltrated patches.

In summary, using two assays we demonstrated that the AGO binding 16K protein of TRV could not inhibit the activity of preassembled RISC complexes.

4.2 Appearance of C-terminally truncated SPMMV P1 proteins *in planta*

It was demonstrated that the N-terminal 383 amino acid region of SPMMV P1 possessed the silencing suppressor domain. This result might not be surprising, since it is already known that maturation of the huge potyviral polypeptide translated occurs via the intrinsic protease activities of the polypeptide. Even P1 has a protease domain in its C-terminal, which is required for liberation of P1 from the primary translation product. Thus, the C-terminal truncation of P1 can be a consequence of its own protease activity, but the action of the plant proteases could not be excluded. However, the primary aim of our study was to whether these shorter versions of P1 still have RNA silencing suppressor activity. To answer

this question, we systematically performed C-terminal truncations on the SPMMV P1 protein. Thus, we prepared the N-terminal 361, 305, 210 and 120 amino acid containing versions of SPMMV P1 named $P1_{1-383}$, $P1_{1-360}$, $P1_{1-305}$, $P1_{1-210}$ and $P1_{1-120}$ respectively. *Agrobacteria* strains harboring these constructs were co-infiltrated with reporter construct GFP-171.1 to check for silencing suppressor activity. SPMMV $P1_{1-383}$ was used as a control, because this is known silencing suppressor. 48 hours post infiltration, visual inspection showed bright green fluorescence for both the control and the shorter versions of P1 proteins indicating that even the $P1_{1-210}$ protein still had a strong RNA silencing suppressor activity. Taken together, we located the silencing suppressor domain of the 759 amino acid SPMMV P1 protein at its N-terminal 210 amino acid region. We propose that this shorter version will be useful for further investigations.

4.3 P1 protein of the *Sweet potato feathery mottle virus* is the closest homologue of SPMMV P1

According to databank searches and previous studies, the P1 protein of the *Sweet potato feathery mottle virus* (SPFMV) (family *Potyvirideae*, genus *Potyvirus*) showed remarkable homology to the P1 protein of SPMMV. Our group has a longstanding interest in better understanding the role of both the viral and cellular WG/GW proteins. Therefore, we decided to isolate and characterize the P1 protein of SPFMV.

Consensus sequence was used to design primers to amplify the corresponding cistrons of SPFMV. To amplify the corresponding cistrons of SPFMV, we used total RNA isolated from SPFMV infected Sweet potato plants, purchased from the German Collection of Microorganisms and Cell Cultures.

We cloned and sequenced the SPMMV P1 and HC-Pro. Pairwise comparison with showed that SPFMV P1 has only one GW/WG domain compared to SPMMV P1. The first WG/GW at position 25 and the surrounding amino acids are well conserved between SPFMV and SPMMV. Moreover, there is a His (H) residue at position 109 and a Tyr (Y) residue at position 139, which correspond to the Trp (W) 101 and 131 in the SPMMV protein.

The deduced amino acid sequence of Hc-Pro revealed a 458 amino acid protein showing 91-97 % homology to the known SPFMV HC-Pro proteins.

We also wanted to check if the SPFMV P1 and HC-Pro had an RNA silencing suppressor activity. To do this, P1 and HC-Pro were cloned into an agrobacterial expression vector, this vector was pSanyi with HA-tag. To make a functional RNA silencing suppressor assay, we used the standard co-infiltration approach

Agrobacteria strain harboring the 35S-GFP as a reporter gene was co-infiltrated with the SPFMV proteins of interest into *N. benthamiana* leaves. We found that SPMMV P1 but not the empty vector strongly suppressed RNA silencing, which could be seen as bright green fluorescence in the infiltrated patches. However, neither SPFMV P1, nor SPFMV HC-Pro suppressed RNA silencing, which could be anticipated from the lack of the green fluorescence at the right sides of the infiltrated leaves.

4.4 Restoring the Argonaute binding domains of SPFMV P1 and functional analysis of the SPFMV P1 mutants

Our results revealed that the SPFMV P1 bearing only one WG/GW motif did not have RNA silencing suppressor activity. Earlier studies on SPMMV P1 showed that at least two WG/GW domain were required for silencing suppressor activity. We hypothesized, therefore, that the sole WG/GW motif in wild type SPFMV P1 might be insufficient for silencing suppressor activity.

The histidine residue at position 109 and the tyrosine residue at position 139 were changed to tryptophan. Thus, three mutants, H109W, Y139W, and H109W/Y139W, were created, resulting in one additional WG/GW motif in the first two mutants, and two additional WG/GW motifs in the third.

Our results showed very low GFP activity infiltrated with H109W and H139W mutants, which could be due to the lack of RNA silencing suppressor activity. Interestingly, the double mutant P1 H109W/Y139W revealed remarkable silencing suppressor activity.

Therefore we can conclude that partial restoration of the possible Argonaute binding domains of SPFMV P1 was not sufficient, but restoration of all possible Argonaute binding domains of SPFMV P1 resulting three in total, did result in RNA silencing suppressor activity of the protein. Here after we named the SPFMV P1 H109W/Y139W double as gain of function mutant.

AGO binding was tested by immunoprecipitations. Small RNA-loaded AGO1 binding ability was detected in the SPFMV H109W/Y139W mutant and WT SPMMV P1 proteins but not in the SPFMV wt or H109W or Y139W mutant P1 protein, thus indicating that silencing suppressor activity strongly correlated with the capability of AGO1 binding. Thus, three WG/GW motifs were absolutely required for SPFMV P1 to gain silencing suppressor activity.

Next we agroinfiltrated Agrobacteria strains harboring SPFMV mutants H109W, Y139W and H109W/Y139W (gain of function mutant) with GFP-171.1 into fully developed *N. benthamiana* leaves. The H109W and Y139W mutants did not support suppression of miR-171 loaded active RISC complexes. However, SPFMV P1 gain of function mutant and the SPMMV P1 inhibited active RISC, as shown by elevated GFP mRNA and protein levels compared to the negative control.

Thus, we concluded that the SPFMV P1 gain of function mutant could have the same working mechanism than the SPMMV P1 RNA silencing suppressor.

5 Discussion

5.1 Understanding the link between physiology and the working mechanism of the 16K silencing suppressor of TRV

TRV 16K inhibited the initiation step of RNA silencing by reducing the silencing associated siRNAs. However, our collaborators found that 16K bound AGO1, AGO2 and AGO4 of *A. thaliana*. Consistent with the result of Martin-Hernandez et al. (2008), we found that 16K differed from P1 of SPMMV. In other words, 16K could not inhibit preassembled miRNA and siRNA RISC complexes, but at least one step of initiation of silencing, which involves AGO binding.

5.2 The minimal silencing suppressor domain of the SPMMV P1

The prototype RNA silencing suppressor inhibiting preassembled RISC is the SPMMV P1 protein, which consists of 759 amino acids. As it was shown before, this big protein in size contains the RNA silencing suppressor domain at the N-terminal part and a protease domain conserved in the *Potyviridae* family. Shorter versions of P1 were found in unrelated experiments implicated the functionality of these proteins. Thus, we narrowed the silencing suppressor domain to the N-terminal 210 amino acids of P1. This truncated protein has full silencing suppressor activity, compared to longer truncated mutants. Functional studies of the WG/GW domains

The SPFMV P1 protein contains only one WG/GW domain, which is located at the very N-terminal of the protein (W25). Our results showed that this protein has no silencing suppressor activity. Thus based on earlier results, we decided to reconstitute the remaining two WG/GW domains. In contrast to SPMMV P1, SPFMV P1 did not show any silencing suppressor activity when the 2nd or the 3rd WG/GW domain was reconstituted separately, but it did have suppressor activity when both of the "missing" WG/GW domains were restored by site directed mutagenesis. At the moment we could not provide a precise explanation for this. One possible explanation could be that in the case of SPFMV, all the WG/GW domains are required for proper folding of the protein.

6 Acknowledgments

I am very grateful to anybody, who helped me during the write of my thesis.

First of all, I would like to give special thank to my supervisor, Dr. Lóránt Lakatos, for the opportunity in the exciting field of RNA silencing.

I would like to thank Prof. Dr. Kemény Lajos, the head of Department of Dermatology and Allergology, University of Szeged, who allowed for my Ph.D. research at the institute.

I am grateful for the friendship of many colleagues during my time in Bay Zoltan Institute (Szeged) for their kind help. I thank Dr. Máté Manczinger, who also contributed to this work.

I would is especially like to thank my parents and friend for their support and encouragement.

This study was supported by the Hungarian Research Fund (OTKA) grants (NN 107787 and NN11024) to LL. KL was found by the European Union and co-financed by the European Social Fund (grant agreement no TÁMOP-4.2.2.A-11/1/KONV-2012-0035 and TÁMOP 4.2.4.A-2013/2-A2-SZJÖ-TOK-13).