Determination of the inhibitory mechanism for the P1 silencing suppressor of the Sweet potato mild mottle virus and characterization of its closest homologue, the P1 protein of the Sweet potato feathery mottle virus

Final Report

RNA silencing is a post-transcriptional gene regulation mechanism conserved in almost all eukaryotes and involved in many essential biological processes, from development, physiological activity to the regulation of abiotic and biotic stress responses by micro RNAs (miRNA), genome defense by 24 nucleotide (nt) long small interfering RNAs (siRNAs) and in particular antiviral defense by 21 nt siRNAs. RNA silencing negatively regulates genes expression [1].

The trigger of RNA silencing can be pri-miRNAs for miRNAs or double stranded (ds) RNAs as replicative forms of plant viruses. Trigger RNAs are processed into si- and miRNAs by the RNAse III type enzymes Dicers, then small RNAs are loaded into the Argonaute (AGO) protein containing protein complexes called RNA induced silencing complex (RISC). This process is referred to as RISC assembly. In RISC, one strand of the small RNA is eliminated resulting the single stranded (ss) small RNA containing RISC complex (active RISC). Active RISC complexes are able to hamper gene expression either by cleaving the target RNA or by inhibiting the translation of the target RNA [2]. Thus, Argonaute (AGO) proteins play a key role in RNA silencing.

Identification and functional analysis of the P1 protein of SPMMV

We showed that in Sweet potato mild mottle virus (SPMMV), type member of the Ipomovirus genus, family Potyviridae), the role of silencing suppressor is played by the P1 protein (the largest serine protease among all known potyvirids) despite the presence in its genome of an HC-Pro protein, which, in potyviruses, acts as the suppressor. Using in vivo studies we have demonstrated that SPMMV P1 inhibits si/miRNA-programmed RISC activity. Inhibition of RISC activity occurs by binding P1 to mature high molecular weight RISC, as we have shown by immunoprecipitation. Our results revealed that P1 targets Argonaute1 (AGO1), the catalytic unit of RISC, and that suppressor/binding activities are localized at the N-terminal half of P1. In this region three GW/WG motifs were found resembling the AGO-binding linear peptide motif conserved in metazoans and plants. Site-directed mutagenesis proved that these three motifs are absolutely required for both binding and suppression of AGO1 function. In contrast to other viral silencing suppressors analyzed so far P1 inhibits both existing and de novo formed AGO1 containing RISC complexes. Thus, P1 represents a novel RNA silencing suppressor mechanism. The discovery of the molecular bases of P1 mediated silencing suppression may help to

get better insight into the function and assembly of the poorly explored multiprotein containing RISC.

In the last few years, several AGO binding proteins were identified. Most of them contains GW/WG (Trp-Gly/Gly-Trp) domains that mediates the interaction with AGO. This group of proteins was named GW/WG proteins after the founding member GW182 protein of human [3]. GW182 in animals bind AGO and mediate the interaction with the polyA binding proteins to repress the translation of the target RNA. In Schizosaccaromyces pombe, transcriptional silencing involves AGO and TAS3 that shows high similarity to animal GW182 proteins [4]. In plants, heterochromatin silencing exploits a couple of GW/WG proteins, such as the second largest subunit of the RNA polymerase IV complex and SPT5 protein [5, 6]. Moreover, the GW/WG protein SUO was identified in Arabidopsis thaliana to be involved in miRNA driven RNA silencing [7]. The above mentioned proteins are required for proper RNA silencing in different organisms. However, our results shed light on the first GW/WG protein that negatively regulates RNA silencing. Our results were published in Plos Pathogens [8].

Towards the RNA silencing mechanism of SPMMV P1

As SPMMV P1 is being an RNA silencing suppressor inhibiting active RISC complexes, we aimed the determination its working mechanism. Our hypothesis was that binding P1 to AGO1 does not allow target RNA binding to AGO1. To test this, we adopted an RNA immunoprecipitation method. P1, AGO1 35S-GFP and a GFP inverted repeat (GFP-IR) was agroinfiltrated and at 3 dpi, cross-linked with formaldehyde. P1 was immunoprecipitated. In the eluate of the IP was positive for P1, AGO1 but not for the target RNA (35S-GFP). As a control, we used the same setup except we used the P1 version, in which all the three GW/WG motifs were mutated. This mutant is not able to bind AGO1, thus the eluate of this IP was positive for the mutant, but not for AGO1, nor target RNA. Thus, we concluded that P1 binding does not allow target RNA binding to AGO1. We have not published this result yet. For reasons, please see below.

Functional analysis and remodeling the GW/WG domains of the P1 protein of SPFMV

Both from the evolutionary and mechanistic point of view, it is an interesting question to study homologous proteins. Using homology searches, we found that the closest homologoue of the SPMMV P1 is the P1 protein of the Sweet potato feathery mottle virus (SPFMV). Degenerated oligonucleotide primers were designed for the P1 of SPFMV and the P1 region was PCR amplified from first strand cDNA generated from RNA isolated from SPFMV infected sweet potato. The amplified PCR product was cloned and the nucleotide sequence was determined. The deduced amino acid sequence of our P1 isolated consists of 689 amino acid and was almost identical to the P1 sequences in the databases. The N-terminal 193-aa region of SPFMV P1 was

41.5% identical to the corresponding part of SPMMV P1; the rest of the protein was 22% identical. However, the overall identity was 24.6%. Sequence analysis also showed that in contrast to SPMMV P1, the SPFMV P1 contained only one GW/WG domain at position 25. Interestingly, SPFMV P1 contains a His residue at position 109 and a Tyr residue at position 139, while SPMMV P1 has Trp residues at the corresponding positions (Figure 1.)

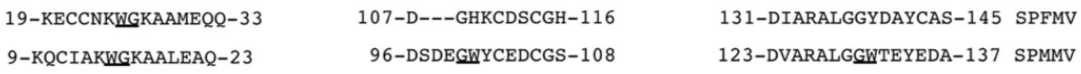

Figure 1. GW/WG motifs of SPFMV P1 and their corresponding SPMMV P1 parts.

Then the silencing suppressor activity of SPFMV P1 was tested. SPFMV P1 was coinfiltrated with 35S-GFP. As a control, SPMMV P1 was used. We found that SPFMV P1 protein did not have any detectable silencing suppressor activity in this assay. Since the GW/WG domain of the SPMMV P1 play an important role in silencing suppressor the activity, we hypothesized that the one and only GW/WG domain of SPFMV P1 is not sufficient to support RNA silencing activity. Therefore, we decided to remodel the GW/WG domain of SPFMV P1 and check the silencing suppressor activity of the mutants. Using site-directed mutagenesis, we changed H109 to W and Y139 to W as well. Thus, three mutants, H109W, Y139W, and H109W/Y139W, were created, resulting in one additional GW/WG motif in the first two mutants, and two additional GW/WG motifs in the third. In a 35S-GFP silencing suppressor assay we found that the H109W and Y139W mutant did not, but the H109W/Y139W mutant did have silencing suppressor activity. Moreover, we tested the mutants and the wild type (wt) SPFMV P1 using the GFP-171.1 reporter construct in agroinfiltration assay. The GFP-171.1 reporter contains a miR-171 target site at its 3 prime UTR and miR-171 is present exclusively in RISC complexes in leaves used for infiltration. Thus, this assay tests silencing suppressor activity against active RISC complexes. In this assay we found that the H109W and Y139W mutant did not, but the H109W/Y139W mutant did inhibit active RISC complexes.

In the case of SPMMV P1 we found that the silencing suppressor activity and the AGO1 binding activity positively correlated. Therefore we tested if the same is true for SPFMV P1. In an agroinfiltration based coimmunoprecipitation assay we found that the H109W and Y139W mutant did not, but the H109W/Y139W mutant did bind AGO1. Our results showed that the remodeled SPFMV P1 functionally and mechanisticly corresponds to the SPMMV P1.

Bioinformatics analysis showed a close evolutionary relationship between the P1 proteins of SPMMV and SPFMV. Although wt SPFMV P1 did not show any silencing suppressor activity, remodeling of the AGO hook by changing only two amino acids to tryptophan resulted in a protein that inhibits active RISC by the same mechanism as the SPMMV P1 prototype. Thus, the close evolutionary relationship between the P1 proteins of SPMMV and SPFMV was further proven by our functional analyses. SPFMV, in a synergistic interaction with *Sweet potato chlorotic*

stunt virus (SPCSV), causes the very severe sweet potato virus disease. RNase3 protein, the silencing suppressor of SPCSV, was found to mediate viral synergism between SPCSV and SPFMV. In such a synergistic interaction, one powerful suppressor can support the spread of two viruses and that might explain why SPFMV P1 did not bear silencing suppressor activity.

Finally, to our knowledge, this is the first instance in which a viral protein of unknown function was turned into a functional RNA silencing suppressor. Or results were published in Journal of Virology [9].

Establishing the full length cDNA clne of SPMMV

To be able to study P1 in a viral in vivo background, we aimed to create the full length infectious cDNA clone for SPMMV. Although we made a great effort to do this, unfortunately, we have not achieved our aim yet. Fortunately, we still have some new approach to get the full cDNA clone.

Identification and characterization of the zinc finger motif in the SPMMV P1

GW/WG motif proteins contain several (2-20) GW/WG domains, which confer AGO binding. Unexpectedly, we found a zinc finger domain in the P1 protein of SPMMV (Figure 2.). As you can see the putative zinc finger domain is a Cys4-type, Cys 88, 91, 103 and 106 residues could be reached by the Zn^{2+} ion.

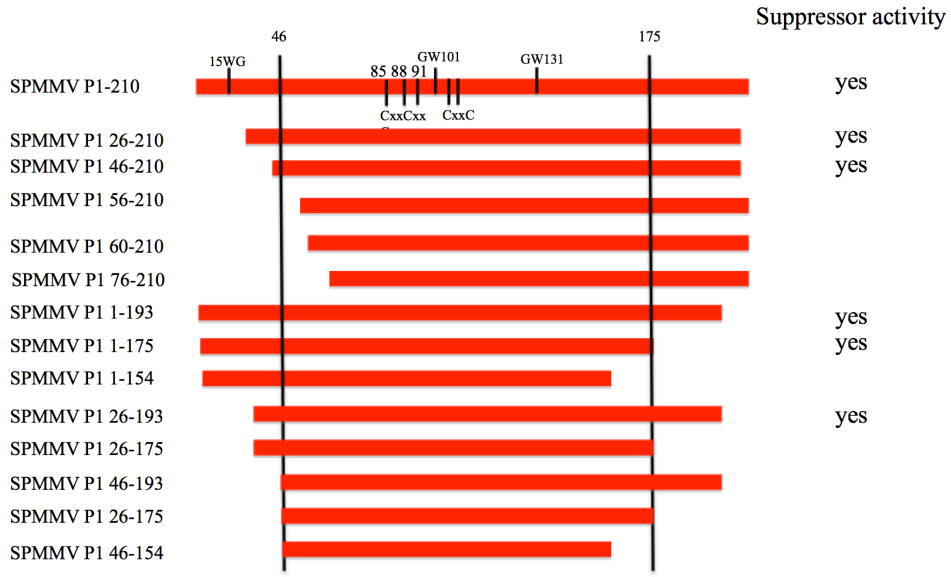

Figure 2. Graphical representation of the zinc finger domain and the truncated mutants of P1

To see, if the zinc finger domain is required for RNA silencing suppressor activity, we mutated Cys residues to Ala creating the Cys85A, Cys88A, Cys91A, Cys85A,88A, Cys88A,91A, Cys85A,91A mutants. Mutants were tested to inhibit

active RISC using the GFP-171 reporter gene. Our results showed that Cys85 is dispensable for the activity. The lack of Cys88 lowered the activity to 50% and Cys91 is indispensable for the silencing suppressor activity.

SPMMV P1 contains GW/WG domains. Mutagenizing 2 out of 3 GW/WG domains led to activity of the protein [8]. To evaluate the requirement for each GW/WG domain, we tested the activity of the P1 mutants having only 2 GW/WG motifs. We found that W15 and 101 were dispensable for activity, but W131 lowered suppressor activity by half. Figure 2. Shows that W101 is surrounded by the Cys residues of the zinc finger domain, which is absolutely required for suppressor activity. Thus, we concluded that the zinc finger domain and W131 together might play important role in the activity of the protein. Therefore, we tested if spacing between the zinc finger and W131 has any role in suppressor function. First we inserted 10 amino acids between the zinc finger and W131 and found that activity changed to the 80% of the wt. When 10 amino acids were deleted between the zinc finger and W131, suppressor activity was completely lost. We concluded that a minimal spacing between the zinc finger and W131 is required for silencing suppressor activity of P1. We also concluded that P1 is the one and only GW/WG protein, whose activity requires a zinc finger domain.

Analysis of the truncated P1 mutants

We systemically truncated P1 protein from the C-terminal end to get a shorter active version. Finally, the first 210 amino acid version of P1 was still active. Having known that W15 is not required for suppressor activity, we started to create both N- and C-terminal truncation mutants of the P1 1-210 protein. To get the shortest active P1 version, we had two aims: i; to be able to distinguish the binding function and silencing suppressor activity, if exists, ii; we aim structural studies of P1, which requires the possibly shortest active protein. As Figure 2. shows, N-terminal truncation resulted in the P1 46-210 mutant, which was still active. The shortest active C-terminal truncation mutant was the P1 1-175. Next, we created the P1 46-175 version, which did not show activity. Finally, the shortest active mutant we got was the P1 26-193 mutant (Lakatos, unpublished).

In the case of the GW/WG mutants of the SPMMV P1, silencing suppressor activity and AGO1 binding positively correlated. We took advantage of the truncated mutants we created to check if they are able to bind AGO1. Physical interaction was checked with agroinfiltration followed by coimmunoprecipitation. Interestingly, we found that the P1 C88A,C91A, P1 60-210 and the P1 76-210 mutants, which were shown not to have silencing suppressor activity, were still able to bind AGO1. Thus, analysis of our new set of mutants allowed us to distinguish between AGO1 binding and silencing suppressor activity.

Moreover, we believe that P1 60-210 and the P1 76-210 mutants can be used to determine the working mechanism. We hypothesized that these N-terminally truncated mutants, which were shown not to have silencing suppressor activity and were still able to bind AGO1, allow the small RNA-target RNA binding. This

hypothesis is being tested now. These promising results with our results on the zinc finger are planned to be published together.

Literature cited

1. Baulcombe D: **RNA silencing in plants**. *Nature* 2004, **431**(7006):356-363.
2. Hutvagner G, Simard MJ: **Argonaute proteins: key players in RNA silencing**. *Nat Rev Mol Cell Biol* 2008, **9**(1):22-32.
3. Pfaff J, Meister G: **Argonaute and GW182 proteins: an effective alliance in gene silencing**. *Biochem Soc Trans* 2013, **41**(4):855-860.
4. Buhler M, Verdel A, Moazed D: **Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing**. *Cell* 2006, **125**(5):873-886.
5. Bies-Etheve N, Pontier D, Lahmy S, Picart C, Vega D, Cooke R, Lagrange T: **RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family**. *EMBO Rep* 2009.
6. Herr AJ, Jensen MB, Dalmay T, Baulcombe DC: **RNA polymerase IV directs silencing of endogenous DNA**. *Science* 2005, **308**(5718):118-120.
7. Yang L, Wu G, Poethig RS: **Mutations in the GW-repeat protein SUO reveal a developmental function for microRNA-mediated translational repression in Arabidopsis**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(1):315-320.
8. Giner A, Lakatos L, Garcia-Chapa M, Lopez-Moya JJ, Burgyan J: **Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs**. *PLoS Pathog* 2010, **6**(7):e1000996.
9. Szabo EZ, Manczinger M, Goblos A, Kemeny L, Lakatos L: **Switching on RNA silencing suppressor activity by restoring argonaute binding to a viral protein**. *J Virol* 2012, **86**(15):8324-8327.