FINAL REPORT

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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 (Baulcombe, 2004).

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 (Burgyan & Havelda, 2011). Thus, Argonaute (AGO) proteins play a key role in RNA silencing.

In the last few years, several AGO binding proteins were identified. Most of them contains WG/GW (Trp-Gly/Gly-Trp) domains that mediates the interaction with AGO. This group of proteins was named WG/GW proteins after the founding member GW182 protein of human. GW182 in animals bind AGO and mediate the interaction with the polyA binding proteins to repress the translation of the target RNA Thus, these WG/GW proteins have a positive effect on RNA silencing and are absolutely required for the efficient carrying RNA silencing into execution.

Two viral proteins bearing WG/GW domains were found to have a negative effect on RNA silencing. The p38 protein of the Turnip crinkle virus sequesters small RNA unloaded AGO1(Azevedo, Garcia et al., 2010). However, the P1 RNA silencing suppressor protein of the Sweet potato mild mottle virus (SPMMV) is markedly different from that of p38. SPMMV P1 binds AGO1 of Arabidopsis thaliana and Nicotiana benthamiana loaded with si- or miRNAs, thus inhibiting target cleavage of active RISC complexes. Mutational analysis revealed three WG/GW motifs resembling the AGO binding platform conserved in plants and metazoans at the N-

terminal part of P1 are required for AGO binding and silencing suppressor activity (Giner, Lakatos et al., 2010). In a separate study we showed that the P1 protein of the Sweet potato feathery mottle virus (SPFMV) -the closest paralogue of the SPMMV P1 does not have silencing suppressor activity, but restoring the AGO binding domain of SPFMV P1 by changing only two amino acids to tryptophan residues resulted in a protein that inhibits active RISC by the same mechanism as the SPMMV P1 prototype (Szabó EZ et al., 2014).

During the course of our work, our aim was

(i) to characterize the SPMMV P1-AGO interaction in detailed and to propose a working mechanism for the P1 silencing suppressor.
(ii) We also planned to carry out crystallographyc studies of the Ago-P1 complex.

Differential effect of P1 on AGO1 and AGO2

An in vivo assay in N. benthamiana was used to analyze the effect of SPMMV P1 on the cleavage and target RNA binding activities of Arabidopsis AGO1 and AGO2, the two main plant antiviral AGOs. We observed that SPMMV P1 inhibits both endogenous and overexpressed AGO1 but not AGO2 cleavage function. Indeed, we noticed that P1 enhanced endogenous AGO2 activity at the transcriptional level, however, AGO2 activity could be induced by P1 derepressing the AGO1/miR403-mediated silencing of AGO2 mRNA (Harvey, Lewsey et al., 2011). Interestingly, P1 could not inhibit either endogenous or overexpressed AGO2-mediated cleavage despite that both proteins seem to interact with each other according to our coimmunoprecipitation experiments. Although the amino acid similarity between Arabidopsis AGO1 and AGO2 is rather low (33%), a common protein domain involved in P1 binding might be included in the sequence of these two AGOs. Pertinent to this context, it is known that AGO proteins contain a conserved pocket for binding sRNAs and Trp residues of the WG/GW proteins (Till, Lejeune et al., 2007) (Elkayam, Kuhn et al., 2012, Hutvagner & Simard, 2008, Schirle & MacRae, 2012). Therefore, it is possible that the conserved WG/GW domains of P1 (Giner et al., 2010) are also involved in P1 interaction with AGO2.

The zinc finger motif in SPMMV P1 uncouples the silencing suppressor activity and AGO1 binding functions of P1

Cys4-type zinc finger motifs are typically present in transcription factors and RNA binding proteins (Brown, Chu et al., 2005). The mutation of residues in this type of zinc finger motifs can lead to suppression of the protein function, as observed with the individual conversion of Cys residues to Ser residues in the adenoviral Cys4 zinc finger-containing E1A transcription factor that abolished mutant transactivation function (Webster, Zhang et al., 1991). Here, a series of SPMMV P1 forms with Cys to Ala mutations in the four conserved residues of a putative zinc finger motif were analyzed. Only SPMMV P1 zinc finger double mutants showed reduced suppressor activity compared to wild-type P1. Zinc finger motifs have been found in other VSRs. For instance, the AC2 protein from Mungbean yellow mosaic virus-Vigna (MYMV) lost transactivator, DNA binding and VSR activity when its zinc finger motif was mutated (Trinks, Rajeswaran et al., 2005). Also, the zinc finger motif in the p14 protein of Beet necrotic yellow vein virus (BNYVV) and of Beet soil-borne mosaic virus (BSBMV) was required for VSR activity and long-distance movement (Chiba, Hleibieh et al., 2013).

The WG/GW domains of SPMMV P1 were shown to be involved in both AGO1 binding and VSR activity (Giner et al., 2010). In contrast, results presented here show that suppressor-deficient P1 double mutants are still able to interact with AGO1 indicating that the VSR and AGO1 binding functions of P1 can be uncoupled. These results also suggest that the zinc finger motif of P1 is indeed an effector domain. Similarly, in proteins including WG/GW domains such as GW182, KTF1, Tas3 and RNA Pol IV, the AGO binding and the effector functions were mapped to different domains (Bies-Etheve, Pontier et al., 2009, Chekulaeva, Filipowicz et al., 2009, El-Shami, Pontier et al., 2007, He, Hsu et al., 2009, Till & Ladurner, 2007, Zipprich, Bhattacharyya et al., 2009). Thus, the modular architecture of proteins including WG/GW domains might explain how these proteins could play positive or negative role in RNA silencing.

Interestingly, a subset of WG/GW proteins also contain zinc finger domains. For example, in the Arabidopsis NERD protein, the reiterated WG/WG domains are separated from the putative zinc finger motif at the C-terminal end of the protein. In another example, the CnjBp protein of the ciliate Tetrahymena thermophila also contains a zinc finger motif in this case surrounded by two reiterated WG/GW domains (Bednenko, Noto et al., 2009). Unfortunately, the biological role of the NERD or CnjBp zinc finger-containing proteins has not been elucidated yet (Bednenko et al., 2009, Pontier, Picart et al., 2012).

Molecular mechanism of P1-mediated inhibition of AGO1 activity

We previously reported that the SPMMV P1 silencing suppressor inhibits pre-assembled

RISC activity by binding to AGO1 via its conserved WG/GW motifs (Giner et al., 2010). Here, we used an in vivo agroinfiltration system in N. benthamiana, followed by protein/RNA immunoprecipitation and Northern, Western and RT-PCR analyses to show that indeed SPMMV P1 blocks target RNA binding to AGO1. This particular mode of action of P1 represents a novel silencing suppression mechanism for a VSR.

We propose a structural model that could explain how SPMMV P1 suppresses AGO1 but not AGO2-mediated silencing (Figure 1). In this model, the zinc finger domain of P1 might compete with target RNA (substrate) for binding to AGO1-sRNA binary complexes. The seed region (nucleotides 2-7) of the sRNA plays a key role in target RNA recognition of human AGO2 (Schirle, Sheu-Gruttadauria et al., 2014). Structural studies have also reported that in human AGO2-miRNA binary complexes, the helix-7 of the AGO2 L-2 domain is inserted between nucleotides 6 and 7 of the of the sRNA that brakes the A-form of the guide RNA leading to the inhibition of target RNA-guide RNA interaction. However, in the trimeric complex representing the target bound state, the helix-7 (which is conserved between animal and plant AGO proteins) is shifted upon seed pairing resulting in the relaxation of the kink (Schirle et al., 2014) (Figure 1A).

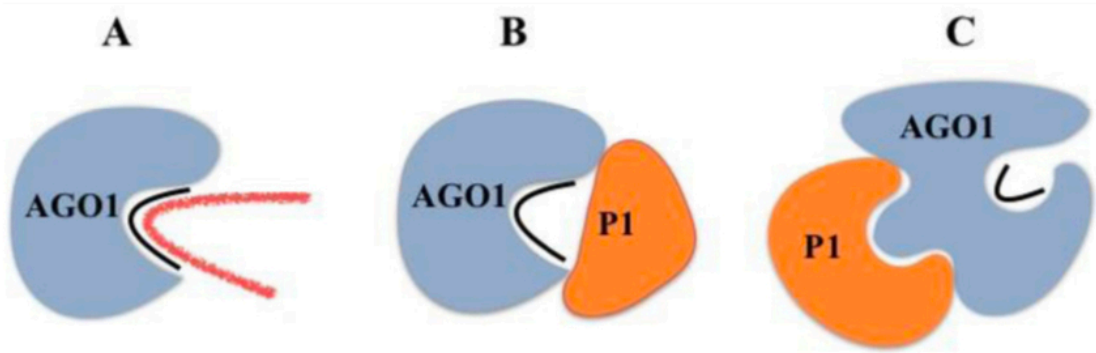

Figure 1. Model for P1 silencing suppression mechanism. (**A**) The AGO1-sRNA binary complex binds target RNA leading to RNA cleavage or translational inhibition. (**B**) P1 interferes with target RNA association in a competitive way. (**C**) P1 interferes with target RNA association in a non-competitive way.

It is possible that the effector domain of P1 does not allow helix-7 displacement by freezing AGO1 in a target unbound state. Alternatively, the P1 zinc finger might cover the first half of the central cleft of the AGO1-miRNA binary complex, which nucleates the seed region of the guide RNA and by making RNA-protein and/or protein-protein interactions in the central cleft,

SPMMV P1 could inhibit target RNA-sRNA interaction (Figure 1B). Finally, P1 could also act in a non-competitive way by altering the conformation of the AGO1-sRNA binary complex which, in turn, could distort the central cleft impeding target RNA binding (Figure 1C).
Considering that P1 zinc finger as the effector domain, the competitive and the non-competitive way of inhibition might be regulated by protein-protein and/or protein-nucleic acid interactions. For example, diverse examples show that zinc finger motifs can promote specific protein-protein interactions to regulate transcription, proteolysis or cellular hypoxic stress (Gamsjaeger, Liew et al., 2007). Regulation of nucleoprotein complexes could also be controlled by protein-nucleic acid interactions either in a nucleotide sequence specific or non-specific ways. For example, the zinc finger protein TFIIIA controls translation in a non-specific way by binding to the sugar-phosphate backbone of the 5S RNA included in the large ribosomal subunit (Brown et al., 2005). Since AGOs bind to the sugar-phosphate backbone of sRNAs (Schirle & MacRae, 2012), it is therefore unlikely that P1 interferes with target RNA association in a non-specific way of RNA binding. In contrast, the zinc finger domain of tristetraprolin protein binds mRNAs by recognizing the AU rich elements at the 3' UTR to facilitate mRNA degradation, thus inhibiting translation of some certain cytokine and chemokine mRNAs (Hall, 2005). The sequence specific inhibition of target RNA association with pre-assembled AGO1 complexes is not likely to completely block AGO1-directed RNA silencing because of the sequence heterogeneity of AGO1-bound sRNAs. In agreement with this idea, we did not observe interaction between P1 and target RNA in our coimmunoprecipitation experiment described in Figure 6. Although P1 bound to both AGO1 and AGO2, P1 could not inhibit AGO2 activity. Therefore, we find more likely that the SPMMV P1 inhibitory mechanism might be based on either competitive or non-competitive way via specific protein-protein interactions (Figure 1B, C).

The functional output of the P1 dependent inhibition of AGO1 in plants is reminiscent to that of the regulation of miRNA-driven RNA silencing by poly(ADP-ribose) polymerases (PARP) (Leung, Vyas et al., 2011). Acute stress increases the poly(ADP-ribosylation) level of AGO1-4 proteins by PARPs. ADP-ribosylation creates a strong negatively charged environment, which might antagonize with target RNA bound to the AGO-miRNA complex leading to the miRNA-target RNA dissociation, or could interfere with target RNA binding by steric hindrance leading to reduced RNA silencing activity (Leung et al., 2011). The mechanism of action of SPMMV P1 seems to differ from that of the poly(ADP-ribosylation) because of the inhibition of AGO function. Hence, the results reported here represent a novel molecular mechanism explaining the inhibition of pre-assembled RISCs.

A model for SPMMV pathogenicity

Single SPMMV infection of sweet potato leaves causes moderate symptoms manifested in mild vein chlorosis and mottling. Symptoms last 2-4 weeks, and SPMMV cannot be detected in newly developed leaves as a consequence of plant recovery from virus infection (Mukasa, Rubaihayo et al., 2006). According to our previous model, we hypothesized that at early stages of SPMMV infections the existing AGO1-miRNA complexes sequester P1 from the de novo vsRNA-containing RISC complexes, leading to mild symptoms and recovery (Giner et al., 2010). In the light of the results presented here, we can postulate a new model that might better explain SPMMV pathogenicity (Figure 2). In this model, SPMMV replication results in vsRNA and P1 protein production. P1 might inhibit viral RNA association with P1-AGO1-vsRNA complexes

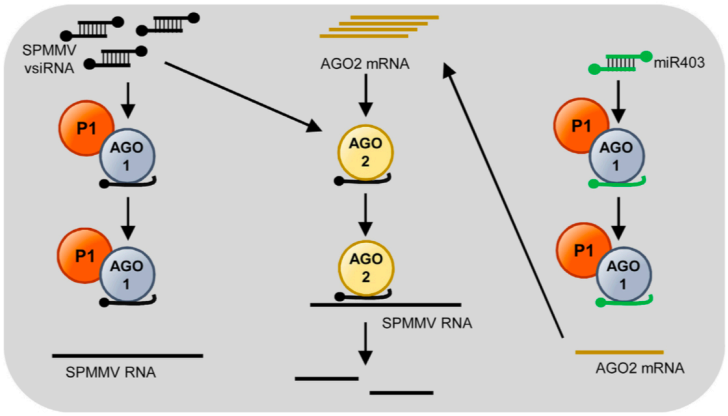

Figure 2. Model for SPMMV pathogenicity based on P1 inhibition of target RNA binding by pre-assembled AGO1 complexes. P1 binding to AGO1 complexes loaded with SPMMV-derived vsiRNAs could inhibit SPMMV RNA targeting by preventing its association with pre-assembled AGO1 complxes. In addition, P1 binding to AGO1/miR403 complexes could prevent their association with AGO2 mRNA binding, leading to derepression of AGO2 mRNA. AGO2 would overaccumulate and load SPMMV vsiRNAs to target complementary SPMMV RNAs. For simplicity, P1 interaction with AGO2 is omitted in this model.

thus preventing viral RNA silencing. In the presence of SPMMV P1, the transcription of the AGO2 mRNA is highly upregulated, which could lead to higher AGO2 protein concentration in infected cells. Although, translation of the AGO2 mRNA is repressed by miR403/AGO1 complexes (Harvey et al., 2011), inhibition AGO1 function by SPMMV P1 might lead to the derepression of miR403/AGO1 mediated silencing of AGO2 mRNA. Thus, the dual regulation of the AGO2 mRNA might account for elevated AGO2 cleavage activity. In this scenario, where AGO1-mediated antiviral silencing is suppressed and AGO2 activity against SPMMV RNA is induced, however, AGO2 could not be inhibited by SPMMV P1 (Figure 2). Thus, AGO2 could be the major player in restricting SPMMV infection and might act as a second defense layer as proposed before for Cucumber mosaic virus and Turnip crinkle virus (Harvey et al., 2011). In

contrast, more recent reports have confirmed that AGO2 is indeed the primary antiviral AGO in certain plant viruses not being targeted by AGO1 such as Tobacco rattle virus (Ma, Nicole et al., 2015) and Turnip mosaic virus (Carbonell, Fahlgren et al., 2012, Garcia-Ruiz, Carbonell et al., 2015). Finally, it seems that SPMMV P1 can bind to AGO2 but somehow it is not able to inhibit AGO2 activity. The biological significance of the P1-AGO2 interaction is still to be determined.

A structural approach to characterize the AGO1- P1 complex

Our biochemical experiments revealed that SPMMV P1 binds both AGO1 and AGO2, however it inhibits only AGO1 activity. Moreover, the zinc finger motif of P1 is essential for P1 activity and considered as an effector domain. Finally, binding P1 to AGO1, P1 excludes the target RNA from the AGO1-small RNA complex to inhibit its activity.

We would support our biochemical results with structural studies to visualize the mode of P1 action on the AGO1 protein.

To do this, we have chosen the baculovirus based expression system, which has the best record to express proteins form eukaryotes.

Because of relatively shorter protein would crystallize more effectively, we determined the shortest version of SPMMV P1, which shows full silencing suppressor activity. Checking the activity of the 360, 305, 210 and 120 amino acid (aa) N-terminal region of P1, we found that the 210 aa protein had full silencing suppressor activity (Szabó EZ et al., 2014). We further trimmed both N- and C-terminally the P1-210 protein to get a rid of the predicted unstructured regions of the protein, which were predicted by bioinformatics. Thus, we found two even smaller regions, such as P1-1-193 and P1- 27-193. P1-27-210 still had comparable RNA silencing suppressor activity.

Interestingly, we also found that the 1-394 amino acid version of P1, which has a very strong RNA silencing suppressor activity, binds more strongly to AGO1-DAH, then the smaller P1 versions. (Kenesi, Carbonell et al., 2017). Moreover, our experiments led us conclude that the AGO1-DAH (catalytically dead) is able to bind more efficiently to P1 in different size (Kenesi et al., 2017).

According to our result, we have chosen AGO1-DAH, P1-27-193 and P1-1-394 for further experiments. To express a protein complex in any expression system is more challenging then to express a single protein. For this reason, we first used the pFastBacDual vector, which contains two polycloning sites. Since our idea was to express AGO1-DAH/P1- 27-193 and AGO1-DAH/P1-1-394, first we cloned AGO1-DAH ORF into one of the cloning site of

pFastBacDual. The second step was to insert N-terminally FLAG-tagged P1-27-193 or P1-1-394 into the second cloning site. Although we made a great effort to complete these tasks, for unknown reasons, the construct could never be recombined into the baculovirus. To achieve our goal, we changed the strategy by cloning AGO1-DAH and the P1 ORFs into two different vectors, pFastBac HTB (no His-tag) for AGO1-DAH and pFastBacDual for the P1 proteins separately. We were aware of the fact that in this case three separate recombinant virus stock instead of two should be prepared. We were also aware of promoter squelching, therefore we used the polyhedrin and the p10 promoters to express AGO1-DAH and P1 proteins. Using this approach, preparation of the constructs and recombination into the baculovirus were all successful. Then recombinant baculovirus DNA was introduced into SF9 cells and in two steps we were able to achieve high titer baculovirus stocks for the P1 constructs (8.6•107/ml for P1-27-193 and 1,03•108/ml for P1-1-394 determined by plaque assay. However, the virustiter for AGO1-DAH was unfortunately about half a magnitude lower (3.4•106/ml) that that of P1. Finally, using small scale SF9 cultures infected with the recombinant baculoviruses, we were able to detect AGO1-DAH and the two P1 protein, which made our work eligible for further experiments.

There are at least two ways to achieve formation of protein complexes for crystallography studies. (i) Expressing proteins separately, then purified proteins will be used for reconstitution of the complex. (ii) Expressing the proteins in the same cells allow us to expect formation of the protein complex.

Using the first approach to reconstitute AGO1-DAH/P1 complexes was totally unsuccessful. Then the co-infection approach was used for further experiments. According to the general rule of heterologous protein expression is that long proteins (140 kDa for AGO1-DAH) could be expressed poorly. Because of insects have a highly efficient RNA silencing system, there is a chance that Arabidopsis AGO1-DAH might be active in SF9 cells having a toxic effect by interfering with the endogenous RNA silencing machinery. Indeed, both P1 proteins were expressed at much higher level, which could be due to their smaller size Moreover, P1 proteins are highly specific for AGO1 proteins, they do not even inhibit Arabidopsis AGO2, which might mean that they do not interfere with the endogenous RNA silencing system of the SF9 cells.

Taking all these facts into consideration, we co-infected SF9 cells AGO1-DAH and P1 in different multiplicity of infection (MOI) as shown in Figure 3.

From the optimization experiment we concluded that in the case of AGO1-DAH/P1-1-395 the 1:1 MOI ratio, but for the AGO1-DAH/P1-27-193 expression the 1:0.1 MOI ratio looked promising (Figure 3).

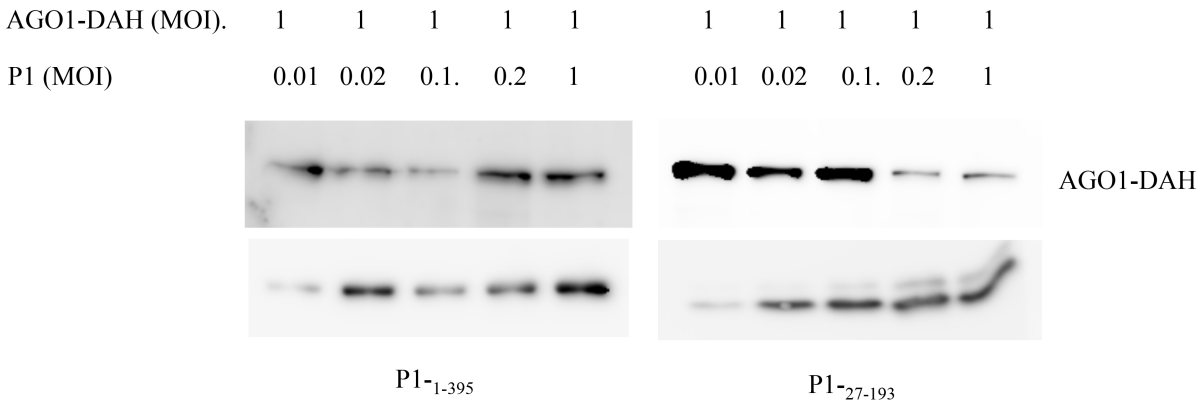

Figure 3. Optimization of co-infection. 50 ml SF9 cells (10^6/ml) was co-infected with AGO1-DAH and P1 expressing baculovirus virions, with the ratio as indicated. After three days of incubation cell were harvested, protein extracts were prepared then AGO1-DAH and P1 proteins were separated on an 8% and 12% SDS PAGE, blotted and detected by anti-AGO1 and anti-FLAG antibodies .

Purification of AGO1-DAH/P1 complexes

For large scale purification, we used 500ml cultures with 5•105/ml SF9 cells at 1:1 MOI for AGO1-DAH/P1-1-395 and 1:0.1 MOI for AGO1-DAH/P1-27-193 as determined earlier. Before SF9 cells started to lysate, cells were collected, and protein extracts were made. To isolate AGO1-DAH/P1-1-395 and AGO1-DAH/P1-27-193 complexes were imunoprecipitated via the FLAG-tagged P1 proteins as in described in Giner et al., 2010; Kenesi et al., 2017. Proteins were eluted with FLAG peptide, then applied into a Superdex HR200 gelfiltration column Figure 4). The AGO1-DAH/P1-1-395 prteins peaked at cca 240 kDa, while AGO1-DAH/P1-27-193 was detected at about 180 kDa.

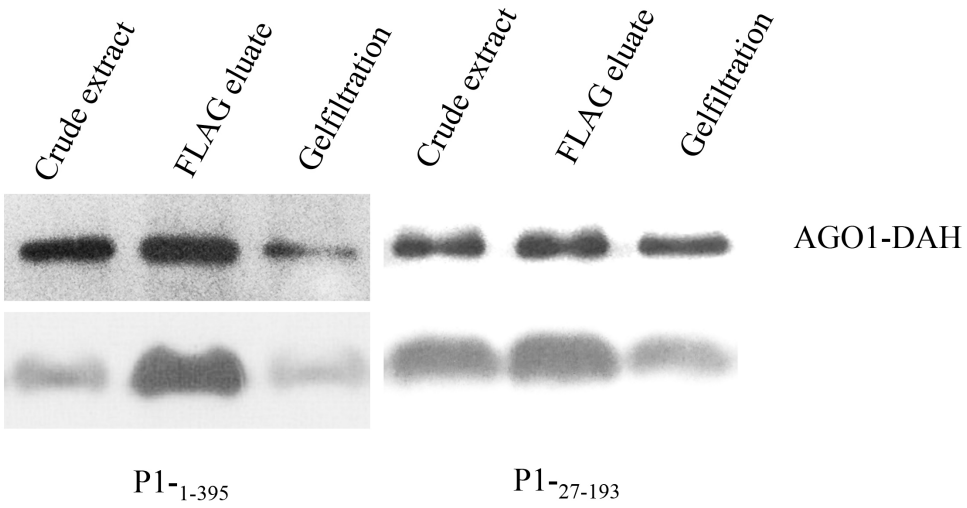

Figure 4. Purification sceme of AGO1/P1 complexes. Crude extracts were immunoprecipitated, eluted, then separated on a gelfiltration column. Peak fractions containing both proteins were collected and combined. Proteins were detected by Western blotting.

The P1 AGO1 interaction was previously demonstrated by immunoprecipitation, but the fact that they could be found in the same fractions in a gelfiltration experiment further strengthens or earlier results (Giner et al., 2010, Kenesi et al., 2017). Combined peak fraction were concentrated on microspin columns. Figure 5 shows the result of the purification of the AGO1-DAH/P1 complexes.

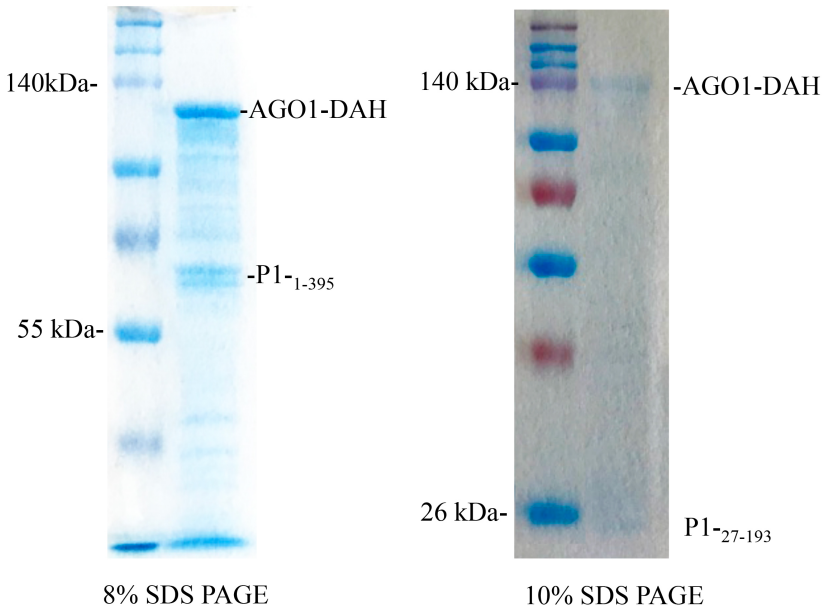

Figure 5. Visualization of the AGO1-DAH/P1 complexes. An aliquot of the concentrated protein complexes were loaded onto SDS PAGE gels, then stained with Brilliant Blue G

We could isolate the AGO1-DAH/P1-1-395 complex in higher amount then the AGO1-DAH/ P1-27-193 complex. However, the AGO1-DAH/P1-1-395 did not seem to be pure. Moreover in this case, the AGO1-DAH protein usually migrated slightly lower than the 140 kDa marker. The smearing might represent the degradation product of the AGO1-DAH protein, which probably occurred during the concentration step. In contrast, the AGO1-DAH/ P1-27-193 complex migrated at the appropriate size, but, we isolated much less amount of complex. Unfortunately, during further purification on an ion-exchange column, the AGO1-DAH/P1-1-395 felt apart.

The purified protein complexes were then used to screen crystallization conditions. Several hundred different conditions have been tried, but unfortunately, we did not get positive results. This might be due to the inpurity of the AGO1-DAH/P1-1-395 complex, and/or the humble amount of complexes we could isolate. Fortunately, in the Biological Reesearch Center I am working now, there will be a possibility to use Cryo Electronmicroscopy for 3D structure determination. We believe that this approach requires less protein for successful structural work.

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