Final Report

RNA silencing is a sequence specific mechanism that regulates gene expression in almost all eukaryotes, and controls key biological processes such as development, heterochromatin formation, stress and antiviral responses. It is triggered by double-stranded (ds) RNA (dsRNA) of different length and origin, which is processed into 21-26 nt small RNA (sRNA) duplexes by an RNase III-type exonuclease of the DICER family. The ARGONAUTE (AGO)-containing RISC (RNA-induced silencing complex) protein complex incorporates one of the strands of the sRNA duplex which serves as guide RNA for RISC to recognize and target highly sequence complementary single-stranded RNA (ssRNA).

Antiviral RNA silencing has been described in worms, insects and plants, as a robust cellular mechanism in charge of clearing infecting viruses. However, diverse proteins interfering with the host antiviral RNA silencing machinery have been identified in insect, plant and fungus-infecting viruses. These proteins, so-called viral suppressors of RNA silencing (VSRs), can block RNA silencing pathways at multiple levels.

Virus-host interaction is an intensively studied field, and different approaches were used in many reports to provide functional data.

We used an approach to study virus-host interactions by analysing the AGO bound small RNA species including viral siRNAs and the miRNAs of the host.

Characterization of the AGO bound small RNAs in virus infected plants

Our aim is to study the viral small RNA species that incorporate into the RISC complexes. For this we planned to use P1 protein of the Sweet potato mild mottle virus. The use of P1 protein for this purpose was based on the fact that it binds AGO1 proteins loaded with both mi- and viral derived siRNAs (Giner, Lakatos et al., 2010), thus using P1 as a "bait", we would be able to isolate small RNAs incorporated into AGO proteins. Originally, we were willing to use a virus vector to express P1 into cells, because this approach would provide us the possibility to use P1 for studying small RNAs loaded into RISC in an adequate viral context (no SPMMV cDNA clone is available yet).

First, we used *Tobacco rattle virus* (TRV) as model. To avoid any possible unwanted effect of the p16 RNA silencing suppressor, we inactivated TRV p16 by site-directed mutagenesis in the agrobacterium construct. To fit P1 into the TRV vector, C-terminally

truncated versions of P1 were created and tested by agroinfiltration for silencing suppressor activity. We found that P1 1-210, 1-305, 1-395, but not the 1-120 amino acid in size versions had silencing suppressor activity (Szabo et al, 2014). Then all active SPMMV P1 versions were cloned into the TRV RNA2 to express recombinant SPMMV P1. Although, the constructs were stable during the cloning procedure and in the agrobacteria strain, but unfortunately, they did not seem to be stable, when expressed in *Nicotiana benthamiana* plants to get TRV virions for inoculation. However, P1 protein could not be detected in the recombinant TRV infected plants, thus, not allowing us to use these recombinant viruses to isolate small RNA loaded RISC complexes.

Then, an *in vivo* assay in *N. benthamiana* was used to analyse 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 (Kenesi et al., 2017). Our results indicated that using P1 as a bait we could expect to get AGO1 and AGO2 loaded with small RNAs.

Furthermore, a putative Cys4-type zinc finger motif was identified in SPMMV P1. A series of SPMMV P1 forms with Cys to Ala mutations in the four conserved residues of a putative zinc finger motif were analysed. SPMMV P1 zinc finger double mutants showed reduced suppressor activity compared to wild-type P1. 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 our showed that suppressor-deficient P1 zinc finger double Cys mutants are still able to interact with AGO1, most probably via its WG/GW domains, 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 (Kenesi et al.,2017). We took advantage of our results that the effector domain mutant P1 (P1mut) retained AGO binding capacity and our results showed that using P1mut as a bait, we could pull down both AGO1 and AGO1-DAH (catalytically inactive mutant, target cleavage never occurs). The result with P1mut and AGO1-DAH is particularly interesting, because AGO1-DAH was loaded with miR173 and we were able to detect its cognate target, the TAS1c mRNA (Kenesi et al., 2017). Of note that

these results were based on overexpression of P1 and AGO proteins in *N. benthamiana* and samples were crosslinked with formaldehyde prior to extract preparation to stabilize the AGO1/miR173/TAS1c complexes. When the crosslinking approach was used during the course our work, crosslinking was always reversed after immunoprecipitation to release RNAs for subsequent steps of the experiment.

Then, we found that expression of the wild type P1 in *N. benthamiana* resulted in the downregulation of the AGO1 protein (Figure 1). Our results were similar to that of the effect of other silencing suppressors to AGO1 protein level (Varallyay & Havelda, 2013). However, as we showed above, the P1mut bearing Csy to Ala mutations in the putative zinc finger domain has weaker silencing suppressor activity, but they still retain AGO1 and AGO2 binding capacity (Kenesi et al., 2017). Interestingly, when we expressed P1mut in *N. benthamiana*, we could detect endogenous AGO1 (Figure 1). Thus, our results revealed that P1mut, but not P1wt might be suitable for isolation of small RNA loaded AGO complexes.

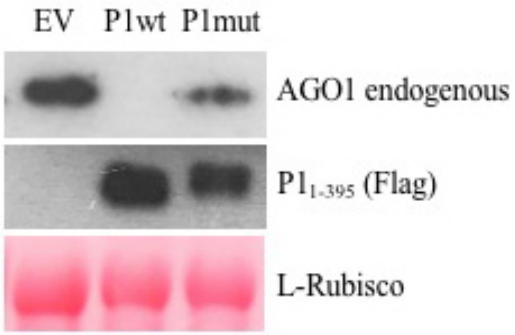

Figure 1. Analysis of AGO1 expression in the presence of P1wt and P1mut. P1 proteins are Flag-tagged and at the N-terminal.

Current model of RNA silencing in plants shows that target RNA cleavage occurs in the cytoplasm and translational inhibition of certain mRNAs in a miRNA dependent way takes place in the endoplasmic reticulum (Li, Liu et al., 2013). Thus, we found of interest to isolate AGO bound small RNA complexes from both the cytoplasm and the endoplasmic reticulum as well. Therefore, we hypothesized that P1mut could be localized in the endoplasmic reticulum as well. We expected this, because AGO1 was found in the endoplasmic reticulum as well (Li et al., 2013) and P1mut has AGO1 binding capacity, as we showed. To test this, we checked the localization of P1mut in infiltrated samples. Cytoplasmic and endoplasmic reticulum fractions were separated by centrifugation, then checked for P1mut and endogenous AGO1 expression. As we expected, P1mut and endogenous were detected in the inputs and in the cytoplasmic and endoplasmic reticulum fractions as well (Figure 2).

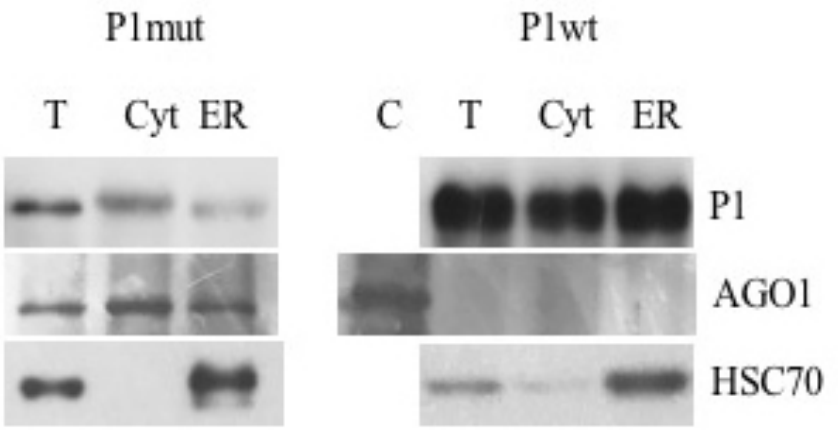

Figure 2. P1mut allows AGO1 expression. T denotes Total extract, Cyt and ER stand for cytoplasmic and endoplasmic reticulum fractions. P1 was detected with the anti-Flag antibody.

To characterize the AGO bound small RNAs, we used the Cymbidium ringspot virus 19stop (Cym19stop) mutant based model, because infection of plants with Cym19stop results in a great amount of virus derived siRNA, and due to the lack of the siRNA binding silencing suppressor protein p19, small RNAs could incorporate into RISC complexes.

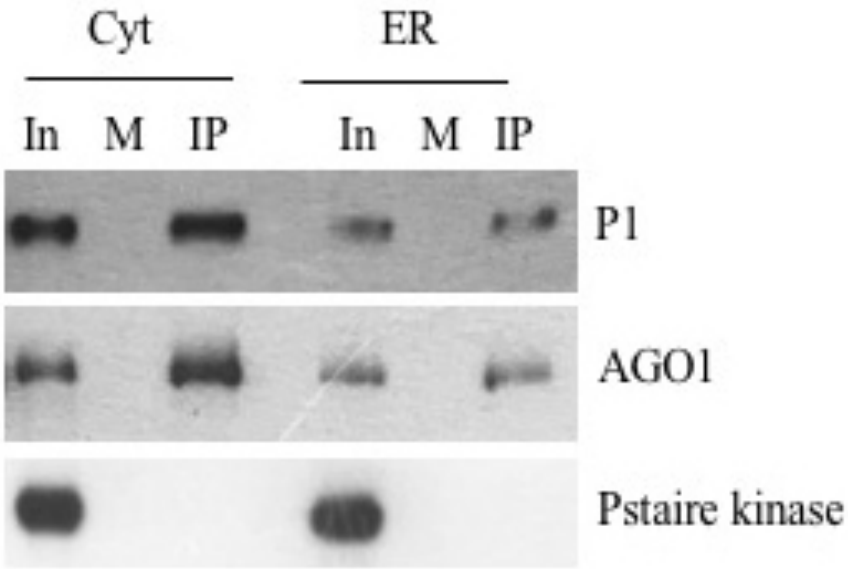

Figure 3. Western analysis of P1 and P1mut immunoprecipitations from fractions of virus infected plants. In denotes Input, Cyt and ER stand for cytoplasmic and endoplasmic reticulum fractions, M is as Mock immunoprecipitation without anti-Flag antibody for P1mut , IP denotes P1 immunoprecipitation with anti-Flag antibody. P1 was detected with the anti-Flag antibody. The Pstaire kinase is localized both in the cytoplasm and the endoplasmic reticulum.

Thus, Cym19stop infected systemic leaves of *N. benthamiana* were infiltrated with P1mut, samples were crosslinked with formaldehyde, the P1mut was immunoprecipitated to

isolate AGO complexes (Figure 3). RNA was isolated from the eluates of immunoprecipitation and used to prepare small RNA libraries. Libraries were sequenced on the SOLID V4 instrument. About 5400 reads from the cytoplasmic faction and 2000read representing viral small RNAs (95 % from the positive strand) could be mapped to the CymRSV genomic RNA. Viral siRNAs were mapped mainly to the 3' end of the virus from both fractions, especially at the third subgenomic promoter, at the very 3' end of the of the p19 and movement protein ORFs, and the 3' end untranslated region of the virus respectively, which is also represented in the p19 and movement protein subgenomic RNAs (Figure 4). However, RNA regions with strong secondary structure are not accessible for RISC mediated RNA cleavage. Having known that the 3'end of CymRSV/Cym19stop contains highly structured regions, we do not believe that small RNAs we detected at the 3' end of the viral RNA could actively take part in antiviral silencing (Schuck, Gursinsky et al., 2013). Moreover, Schuck et al., (2013) found viral siRNAs that could efficiently restrict the replication of a DI-RNA of TBSV. Secondary structure prediction of the target DI-RNA revealed that the binding site for the guide strand of the active siRNA is located at a region, in which the 3' end of the target RNA forms a bulge, which allows target RNA-small RNA interaction (Schuck et al., 2013). We determined the secondary structure of the RNA sequences of the putative binding sites, but unfortunately, we did not get any hit resembling to those siRNA-target RNA interactions. Our result could be explained either there are a few silencing competent viral siRNA exist, or the approach we used was not sensitive enough to find such viral siRNAs. We believe that the latter explanation is more likely, because the 5400 siRNAs we identified from the cytoplasmic fraction, does not cover even twice the genome of CymRSV, and we found gaps, which were not represented at all. Although deep sequencing of formaldehyde fixed animal samples was successful (Meng, McElroy et al., 2013), but this approach used to characterize the expression level of miRNAs from total RNA. In contrast, our studies planned to isolate AGO bound small RNAs and the use of formaldehyde might reduce the efficiency to get an appropriate pool of siRNAs. Also, the concentration of P1mut and/or AGO1 could not be high enough for higher efficiency. Our explanation is supported by our results, that very few reads (178 and 92) were identified as miRNAs in the cytoplasmic and endoplasmic fractions.

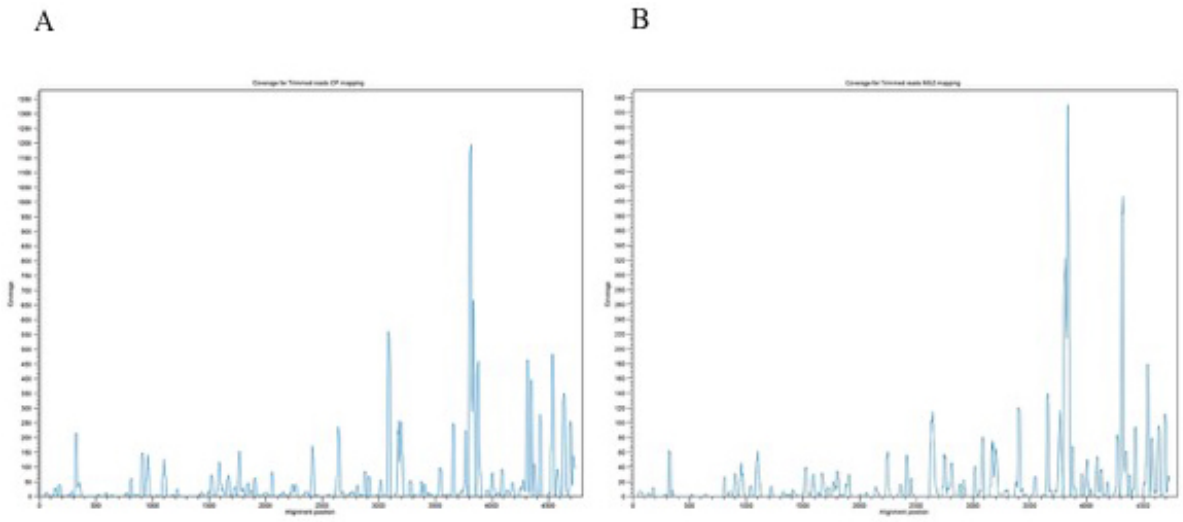

Figure 4. Mapping of viral siRNA reads to the genome of CymRSV. (A)Y axis is scaled as 1-1350. (B) Y axis represents reads 1-540.

We finally approached the characterization of small RNAs in the cytoplasmic and endoplasmic reticulum fractions by Solexa sequencing. Initial bioinformatics analysis revealed an average of 1000 times more reads. About 90 % of the reads derived from the virus and we detected at least 150 different miRNAs.

In our opinion, our idea was not entirely unrealistic, because this year a method resembling our approach was published (Hauptmann and Meister, 2017). Mammalian AGO proteins together with the members of the GW182 proteins are required for translational inhibition in animals. GW182 proteins, like SPMMV P1, interact with AGO with the Tryptophan (W) residues located in their WG/GW motifs. Thus, peptides representing WG/GW motifs of GW182 were expressed bacterially in fusion with the GST-tag, immobilized on the appropriate resin and was used a bait to fish out functional RNA silencing machineries. Therefore, this approach could be used to isolate AGO complexes from human cell extracts and theoretically from protein extracts of any animal origin. Similarly, we also expressed P1wt (1-395 aa.) and used as a bait to isolate AGO complexes from plant extracts, but our effort was not successful.

Biochemical characterization of AMP1, the plant protein involved in miRNA dependent translational inhibition

MiRNA driven translational inhibition in plants involves, at least partly, the AMP1 protein (Li et al., 2013). AMP1 interacts with AGO1 and keeps away certain mRNAs from translation at the endoplasmic reticulum in a miRNA dependent way (Li et al., 2013). First, we asked the question if AGO2 is involved in AMP1 dependent translational inhibition by testing the interaction between AGO2 and AMP1. Our immunoprecipitation experiment revealed that unlike AGO1, AGO2 could not interact with AMP1. In agreement with our results, previously it was shown that the target cleavage activity of AGO2 is required for its antiviral function (Carbonell, Fahlgren et al., 2012), therefore the involvement of AGO2 in inhibition of translation in the AMP1 dependent way is unlikely (Figure 5).

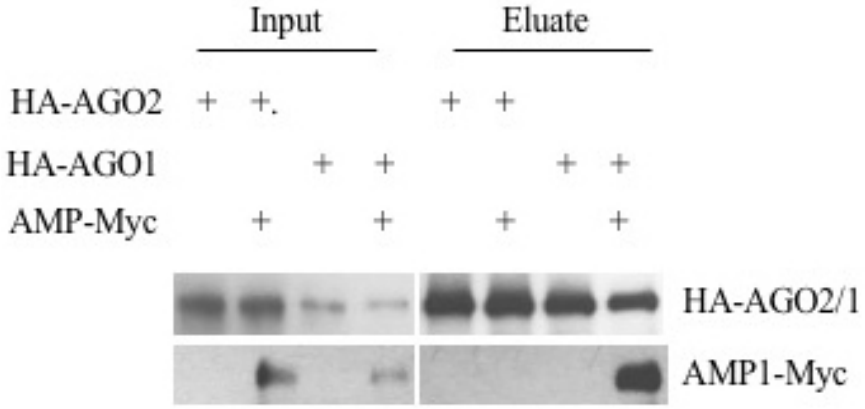

Figure 5. AGO2 does not interact with AMP1. Constructs were infiltrated, as indicated.

Then, our bioinformatics analysis identified three putative WG/GW motifs at amino acid positions 253, 389 and 541 in the AMP1 protein. Conserved WG/GW motifs have been found in almost all eukaryotes and required for AGO binding (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). Moreover, we previously found that the P1 RNA silencing suppressor protein of SPMMV contains 3 conserved WG/GW domains, which are necessary for AGO1 binding (Giner et al., 2010). To test if the WG/GW domains of the AMP1 protein play a role in AGO1 binding, we created Trp to Ala mutations individually in all three putative WG/GW motifs and the resulting mutants were tested for AGO1 binding. Our results revealed that mutation in the third WG/GW domain (amino acid

position 541) reduced AGO1 binding (Figure 6). We concluded that the AMP1 protein is a *bona fide* WG/GW protein. Of note that LAMP1 (Like AMP1) protein was found in *Arabidopsis thaliana* (Li et al., 2013) possessing two putative WG/GW domains that correspond to the first and third WG/GW domain of the AMP1 protein.

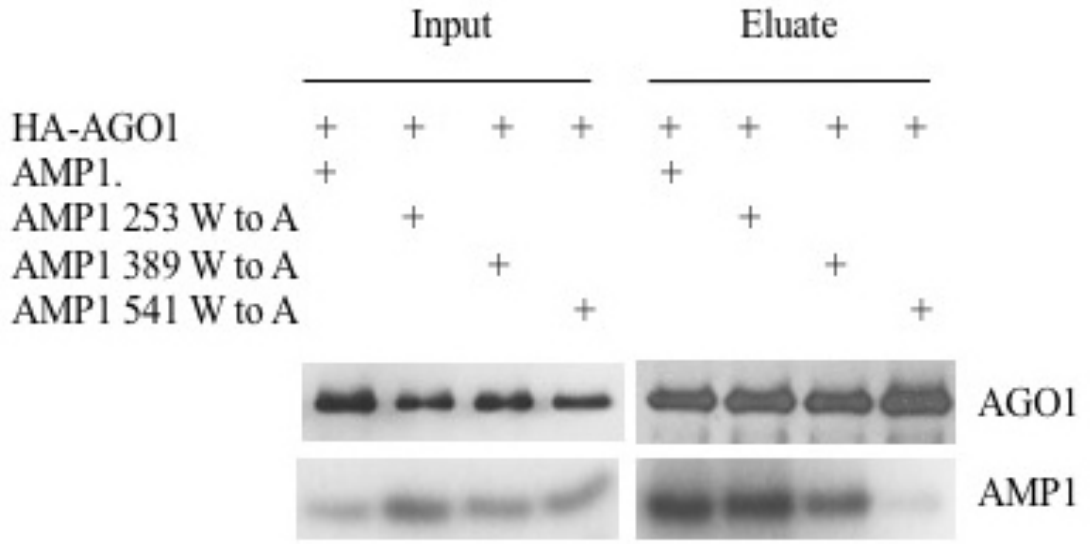

Figure 6. The W residue at 541 position of AMP1 is involved in AGO1 binding. Constructs were infiltrated, as indicated.

AMP1 was solely found in the endoplasmic reticulum of *A. thaliana* cells. The lack of AMP1 protein resulted in increased loading of some certain mRNAs to the ribosomes localised in the endoplasmic reticulum. Specific interaction of AMP1 with AGO1 indicated that AMP1 could inhibit translation in the endoplasmic reticulum in a miRNA dependent way (Li et al., 2013). AMP1 dependent regulation of translation should operate on intact mRNAs , thus, we hypothesized that the miRNA driven target cleavage activity of AGO1 might be regulated by AMP1.

To test this, we have chosen an *in vitro* approach. The AGO1 and AMP1 proteins of *A. thaliana* were expressed in the baculovirus expression system in insect cells. Proteins were purified via the His-tag by affinity chromatography (Figure 7A and B). Then, target cleavage activity of the purified AGO1 was tested. For the cleavage assay, we chose the miR173 double stranded siRNA with its radiolabelled cognate target RNA TAS1c, because the base-pairing between the guide strand of miR173 and TAS1c is 100 %, which definitively directs target cleavage. As expected, in the presence of the miR173, specific AGO1 activity was detected (Figure C, lane 2).

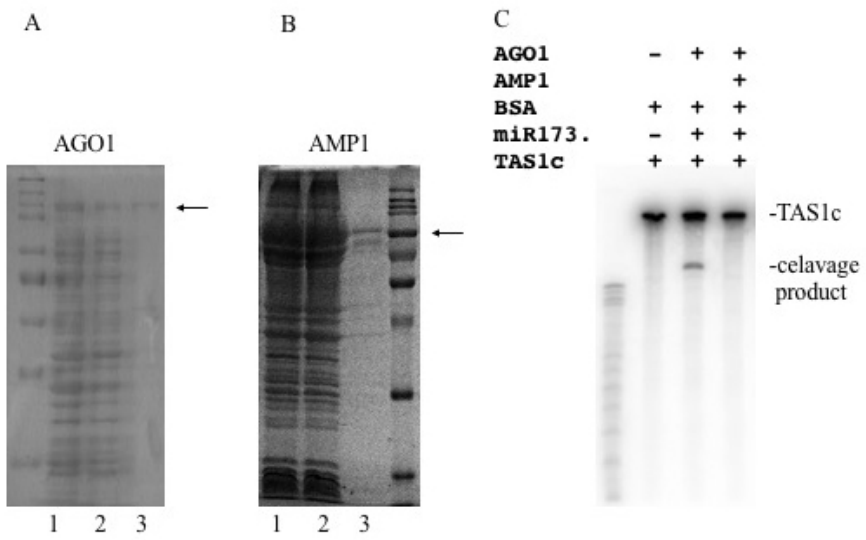

Figure 7. AMP1 inhibits target cleavage activity of AGO1. (A) Purification of AGO1. Lanes 1, 2 and 3 represent total extract, soluble proteins and purified protein. (B) Purification of AMP1. The shorter version of AMP1 lacking the first 50 amino acids were used to avoid membrane localization. Lanes 1, 2 and 3 represent total extract, soluble proteins and purified protein. Arrows indicate purified AGO1 and AMP1 proteins. (C) Test for the effect of AMP1 to the target cleavage activity of AGO1.

However, administration of the purified AMP1 clearly inhibited AGO1 dependent target cleavage activity (Figure 7C, lane 3). Posttranslational regulation of AGO proteins could occur in several ways, such as 4-hydroxylation, methylation, ubiquitin-proteasome dependent degradation (Johnston & Hutvagner, 2011), poly(ADP-ribose) polymerases (PARP) (Leung, Vyas et al., 2011), and viral silencing suppressors. In human, four AGO proteins exist and only AGO2 has target cleavage activity. However, target cleavage activity of human AGO2 is regulated by the level of base-pairing between the guide strand of the small RNA and the target RNA. High level base-pairing facilitates target cleavage, while base-pairing between only the 5' end of the guide RNA and 3' end of the target site directs translational inhibition.

However, in plants, AGO1 dependent target cleavage is not affected by base-pairing between the guide RNA and target RNA as much as in mammals. Therefore, our finding represents a novel layer in regulation of AGO activity in plants.

Based on our *in vitro* results, we hypothesized, if AMP1 inhibits AGO1 cleavage activity, the AMP1-AGO1 complexes might contain endogenous mRNA targets *in vivo*.

To test our hypothesis, we agroinfiltrated a C-terminally tagged version of the AMP1 protein into the leaves of *N. benthamiana*. Then leaves were crosslinked with formaldehyde to fix RNA-protein complexes. Leaf extracts were separated to get cytoplasmic and endoplasmic reticulum fractions and the endoplasmic reticulum fraction were used to immunoprecipitate AMP1-AGO1 ribonucleoprotein complexes via AMP1. The level of AGO2 mRNA might be controlled at the level of transcription (see above), but could be also regulated posttranscriptionally.

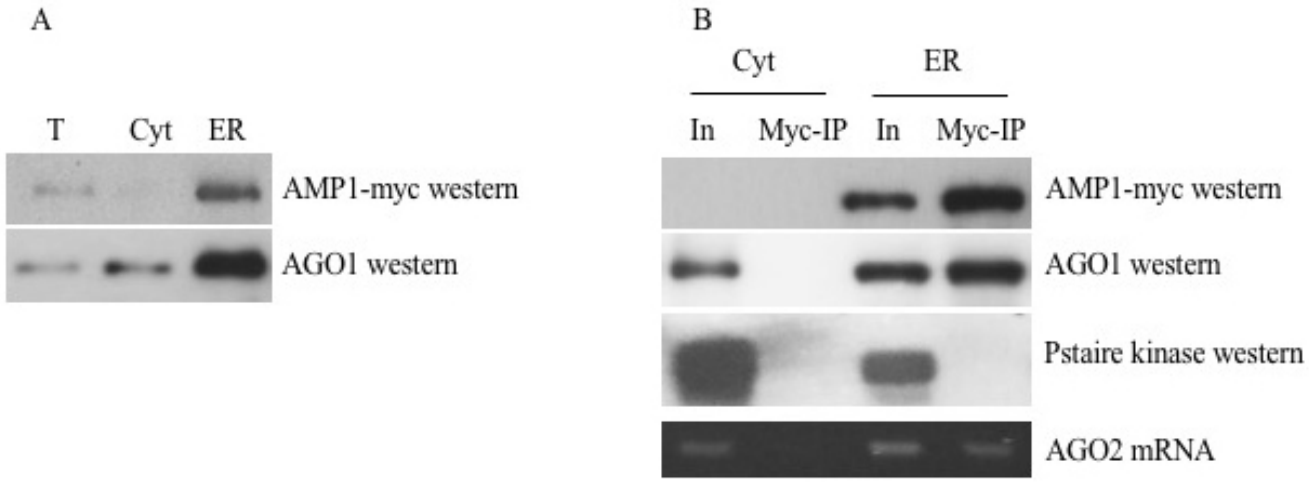

Figure 8. AGO2 mRNA is regulated by the AGO1 AMP1 fashion at the level of translation. (A) Infiltrated AMP1 solely localized in the endoplasmic reticulum. (B) Presence of AGO2 mRNA in the AGO1-AMP1 complex.

Therefore, eluates of IPs were tested for AGO2 mRNA and the presence of Myc-tagged AMP1 and endogenous AGO1 by semi-quantitative RT-PCR (Kenesi et al., 2017). Our results showed that the 3' end of the AGO2 mRNA surrounding the miR403 target site was specifically amplified suggesting the regulation of AGO2 mRNA by active translational inhibition. Although the AGO2 mRNA was also present in the cytoplasmic fraction, no AGO2 specific PCR product could be amplified indicating that AGO2 mRNA might be cleaved by miR403 in the cytoplasm. To justify our RNAIP experiments, the amplified PCR product were cloned and sequenced. Our result showed that all PCR products corresponded to the region of the AGO2 mRNA we were willing to amplify.

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