STUDY OF THE MOLECULAR MECHANISMS OF ANTIVIRAL RNA SILENCING IN SOLANACEOUS PLANTS

K124705 Project Final Report

2022, September

Károly Fátyol

Institute of Genetics and Biotechnology

Hungarian University of Agriculture and Life Sciences

The main objective of the K124705 project was the better understanding of the role of RNA silencing in the antiviral defense in solanaceous plants. Work carried out in the framework of the project mainly focused on the role of the AGO2 molecule, which we and others had previously identified as a crucial component of plant antiviral responses (1, 2). Research supported by this grant is in general conforms the proposed Research Plan. Some deviations are due to new results published by other groups working in the field, delay caused by the lockdown of our institute during the 2020 COVID pandemic, and some unexpected technical difficulties.

OUR PRINCIPAL ACHIEVEMENTS ARE THE FOLLOWING:

1. Identification of the double-stranded RNA binding protein 2 (DRB2) as an important component of virus-induced systemic necrosis.

2. Establishment of a *RDR6* mutant *Nicotiana benthamiana* plant line for studying the role of secondary siRNAs in plant antiviral responses and developmental processes.

3. Unraveling the differential contribution of *AGO1* homeologues of *N. benthamiana* to antiviral defense and development.

4. Uncovering the role of calcium signaling in the regulation of the expression of genes involved in RNA silencing.

5. Understanding the role of abscisic acid signaling in symptom development during virus infection.

6. Unraveling of the role of *AGO2* and *RDR6* in the systemic infection of Citrus Tristeza Virus (CTV).

7. Uncovering the antiviral role of *AGO2* in tomato ringspot virus infected plants.

8. Identification of putative novel components of virus-induced systemic necrosis using comparative transcriptome analyses of wild-type and *ago2 N. benthamiana* plants.

9. Identification of functionally important regions of AGO2.

10. Establishment of *ago2* mutant tomato lines.

11. Uncovering the antiviral role of *AGO2* and *RDR6* in PepMV infected *N. benthamiana* plants.

Details of the above findings are discussed below.

1. Identification of the double-stranded RNA binding protein 2 (DRB2) as an important component of virus-induced systemic necrosis.

Double-stranded RNA (dsRNA) is a common pattern formed during the replication of both RNA and DNA viruses. Perception of virus-derived dsRNAs by specialized receptor molecules leads to the activation of various antiviral measures. We showed that dsRNA-binding protein 2 (DRB2) plays a direct role in systemic necrosis elicited by potato virus X (PVX) in *ago2* mutant *N. benthamiana*. In addition, our findings suggest that *RDR6*-dependent dsRNAs play an important role in the triggering of PVX-induced systemic necrosis. Based on our data, a model is formulated whereby competition between different DRB proteins for virus-derived dsRNAs helps establish the dominant antiviral pathways that are activated in response to virus infection. These results have been published in *Journal of Virology* (3).

2. Establishment of a RDR6 mutant N. benthamiana plant line for studying the role of secondary siRNAs in plant antiviral responses and developmental processes.

Antiviral RNA silencing is triggered by viral dsRNAs of various origins (replicative intermediates, genomic intra-molecular fold-back structures). These molecules are processed into primary viral small interfering RNAs (vsiRNAs) by DCLs. The antiviral response is amplified by the actions of endogenous RNA-dependent RNA polymerases (RDR6 in *N. benthamiana*), which synthesize dsRNAs using aberrant single-stranded (ss) viral RNAs as templates. These dsRNAs serve as substrates for the production of secondary vsiRNAs. Both classes of vsiRNAs are eventually incorporated into AGO containing antiviral RISCs, which can inhibit virus replication via a variety of mechanisms. To unravel the role of the two different classes of vsiRNAs in the antiviral activity of AGO2, we decided to inactivate the *RDR6* gene of *N. benthamiana*. In this species, functional studies of *RDR6* has so far been depended on RNAi based methodologies. These techniques however have inherent limitations, especially in

the context of antiviral RNA silencing. To overcome this issue, we created *rdr6* mutant *N. benthamiana* by the CRISPR/Cas9 genome editing system. Using the mutant, most of the proposed functions of *RDR6* was confirmed. Additionally, the *rdr6 N. benthamiana* plant recapitulated closely the phenotype of the equivalent *Arabidopsis* mutant. In summary, the *rdr6 N. benthamiana* we created may be employed as a model system not only for the better understanding of the role of *RDR6* in pathogen elicited immune responses, but in various developmental processes as well. A paper describing our findings have been published in *Virology* (4).

3. Unraveling the differential contribution of AGO1 homeologues of N. benthamiana to antiviral defense and development.

N. benthamiana has contributed greatly to unravelling antiviral RNA interference, and can also be regarded as an adequate model for studying viral diseases of solanaceous crops. This species, however, as with many of its relatives, possesses an allopolyploid genome, in which homeologous gene pairs frequently occur. *AGO1* is a pivotal component of most plant RNA silencing pathways. The *N. benthamiana* genome encodes two highly similar *AGO1* homeologues: *AGO1A* and *AGO1B*. To understand their roles in planta, their genes were selectively inactivated. Given the inherent limitations of RNA interference-based techniques, we used genome editing to achieve this goal. We found that *AGO1A* was not required for normal development, while *AGO1B* was indispensable for that. By contrast, the two homeologues both contributed to antiviral defense. Additionally, we observed that AGO1B utilized miR168 poorly, which may help to retain a significant level of antiviral RNA interference during viral infection. Our results have important implications for the better understanding of viral diseases of economically important solanaceous crops. The above findings have been published in *New Phytologist* (5).

4. Uncovering the role of calcium signaling in the regulation of the expression of genes involved in RNA silencing.

RNA interference (RNAi) is an across-kingdom gene regulatory and defense mechanism. However, little is known about how organisms sense initial cues to mobilize RNAi. In a collaborative project with Dr. Yule Liu's group at the Tsinghua University, Beijing, we showed that wounding of *N. benthamiana* cells during virus intrusion activates RNAi-related gene expression through calcium signaling. A rapid wound-induced elevation in calcium fluxes triggers calmodulin-dependent activation of calmodulin-binding transcription activator-3

(*CAMTA3*), which activates *RDR6* and Bifunctional nuclease-2 (*BN2*) transcription. BN2 stabilizes mRNAs encoding key components of RNAi machinery, notably AGO1/2 and DCL1, by degrading their cognate miRNAs. Consequently, multiple RNAi genes are primed for combating virus invasion. Calmodulin-, CAMTA3-, or BN2-knockdown/knockout plants show increased susceptibility to geminivirus, cucumovirus, and potyvirus. These findings link Ca^{2+} signaling to RNAi and reveal versatility of host antiviral defense and viral counter-defense. This work has been published in *Cell Host & Microbe* (6).

5. Understanding the role of abscisic acid signaling in symptom development during virus infection.

In collaboration with Dr. Juan Antonio Garcia's group at the Centro Nacional de Biotecnología, Madrid we studied how the different replication strategies employed by various virus groups influence symptom development during viral infection. The genus potyvirus comprises plant viruses characterized by RNA genomes that encode large polyproteins led by the P1 protease. A P1 autoinhibitory domain controls polyprotein processing, the release of a downstream functional RNA-silencing suppressor, and viral replication. We showed that P1Pro, a plum pox virus clone that lacks the P1 autoinhibitory domain, triggers complex reprogramming of the host transcriptome and high levels of abscisic acid (ABA) accumulation. A meta-analysis highlighted ABA connection with host pathways known to control RNA stability, turnover, maturation, and translation. Transcriptomic changes triggered by P1Pro infection or ABA showed similarities in host RNA abundance and diversity. Genetic and hormone treatment assays showed that ABA promotes plant resistance to potyviral infection. Overall, our findings, which have been published in *Plant Communications*, establish a direct link between ABA signaling and plant antiviral immunity (7).

6. Unraveling of the role of AGO2 and RDR6 in the systemic infection of Citrus Tristeza Virus (CTV).

Citrus tristeza virus (CTV, *Closterovirus*) is one of the most complex plant RNA viruses and is an important pathogen of plant species belonging to the *Citrus* genus. As with all RNA viruses, CTV exists as a population of sequence variants that are distributed around a predominant consensus genomic sequence. Based on comparison of consensus genomic sequences, at least seven CTV strains have been distinguished. The symptoms caused by these viral strains are highly diverse, the cause of which is still only partially understood. With the collaboration of Dr. James Ng's group at the University of California, Riverside we determined

that the T36CA strain of CTV (a Californian isolate of CTV) can only establish systemic infection in *N. benthamiana* plants, which are defective in components of RNA silencing (*AGO2* or *RDR6*). These new results establish the important role of RNA silencing in anti-CTV defense. Our findings have been published in *Molecular Plant Pathology* (8).

7. Uncovering the antiviral role of AGO2 in tomato ringspot virus infected plants.

In collaboration with Dr. Hélène Sanfaçon's group at the University of British Columbia, Vancouver we studied the involvement of *AGO2* in the defense against tomato ringspot virus (ToRSV) infection. Previously it was shown that *AGO1* is required for the temperature-dependent symptom recovery of *N. benthamiana* infected with the ToRSV-Rasp1 isolate at 27 °C. It was also demonstrated that symptom recovery from a different isolate, ToRSV-GYV shares similar hallmarks of antiviral RNA silencing but occurs at a wider range of temperatures (21-27 °C). At 21 °C, an early spike in AGO2 mRNAs accumulation was observed in plants infected with either ToRSV-Rasp1 or ToRSV-GYV but the AGO2 protein was only consistently detected in ToRSV-GYV infected plants. Symptom recovery from ToRSV-GYV at 21 °C was not prevented in an *ago2* mutant or by silencing of *AGO1* or *AGO2*. We conclude that other factors (possibly other *AGO*s) contribute to symptom recovery under these conditions. Our results, which also highlight distinct expression patterns of *AGO2* in response to ToRSV isolates and environmental conditions, were published in *Virology* (9).

8. Identification of putative novel components of virus-induced systemic necrosis using comparative transcriptome analyses of wild-type and ago2 N. benthamiana plants.

PVX infection elicited apical necrosis in the *ago2* mutant *N. benthamiana* plants, reminiscent of systemic necrosis. To obtain a deeper insight into the PVX-induced necrotic process, we carried out comparative transcriptome analyses of uninfected and PVX-infected wild-type (w.t.) and *ago2* plants. First, we compared the gene expression profiles of the two plant genotypes (w.t. vs *ago2*, uninfected and infected) (Fig. 1). In uninfected plants (mock), the lack of AGO2 resulted in the differential expression of only 1.9% of the annotated genes. Following PVX infection, the portion of differentially expressed genes increased to more than 5-fold (11,3%), consistent with the specific role of AGO2 in antiviral responses. Next, we looked at the expression changes caused by PVX infection in the same genotype of plants. Approximately 1/3rd of the genes exhibited altered expression following virus infection in both w.t. and *ago2* plants. Half of these genes was upregulated while the rest was repressed. Venn analysis showed that the great majority of these genes were significantly induced (7642) or

repressed (7851) in both *ago2* and w.t. plants following PVX infection. The number of uniquely regulated genes were much smaller. In ago2 plants 1858, while in w.t. plants 836 unique genes were significantly induced after PVX infection. The number of significantly repressed unique genes were 2190 and 1046 in *ago2* and w.t. plants, respectively. Conceivably, these groups of uniquely regulated genes include candidates, which may contribute to the observed hypersusceptibility of *ago2* plants to PVX infection.

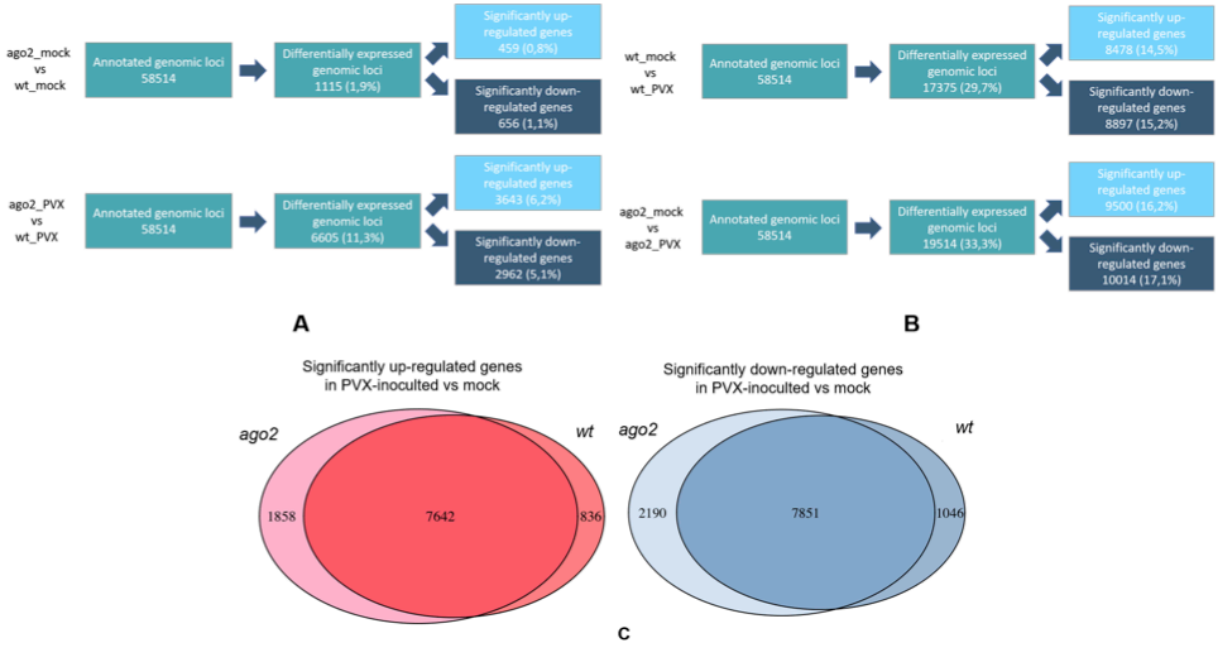

Figure 1. Differential gene expression analysis of uninfected and PVX infected *w.t.* and *ago2 N. benthamiana* plants. See details in the text.

Survey of the differentially upregulated genes identified several promising candidates for further analyses e.g. glutathione S-transferase U8, WRKY transcription factor 6, NAC domain containing transcription factor 2, ethylene responsive transcription factor 1A, glucan endo-1,3-beta-glucosidase, several MAP kinases, ubiquitin ligases and calcium binding proteins. Differential expression of these genes was validated by quantitative real-time RT-PCR. Next, we also tested their expression in plants infected with viruses other than PVX, such as PepMV and TCV. Importantly, unlike PVX these two viruses do not elicit systemic necrosis in *ago2* mutant *N. benthamiana*. Based on the expression patterns two distinct groups of genes could be established. The genes in group 1 are induced upon infection with all three viruses (PVX, PepMV and TCV). This group included glutathione S-transferase U8, WRKY transcription factor 6, NAC domain containing transcription factor 2, ethylene responsive transcription factor 1A, glucan endo-1,3-beta-glucosidase and calcium binding proteins. The genes in group 2 are induced only in PVX infected plants. This group includes two kinases (MKKK15 and OPBK) and an F-box protein (FBP-1) (Fig. 2). While genes in group 1

conceivably contribute to the general stress responses associated with virus infection, genes in group 2 may be specifically involved in PVX-induced systemic necrosis. To test this hypothesis, we selected two genes from each group and initiated their inactivation using CRISPR/Cas9 genome editing.

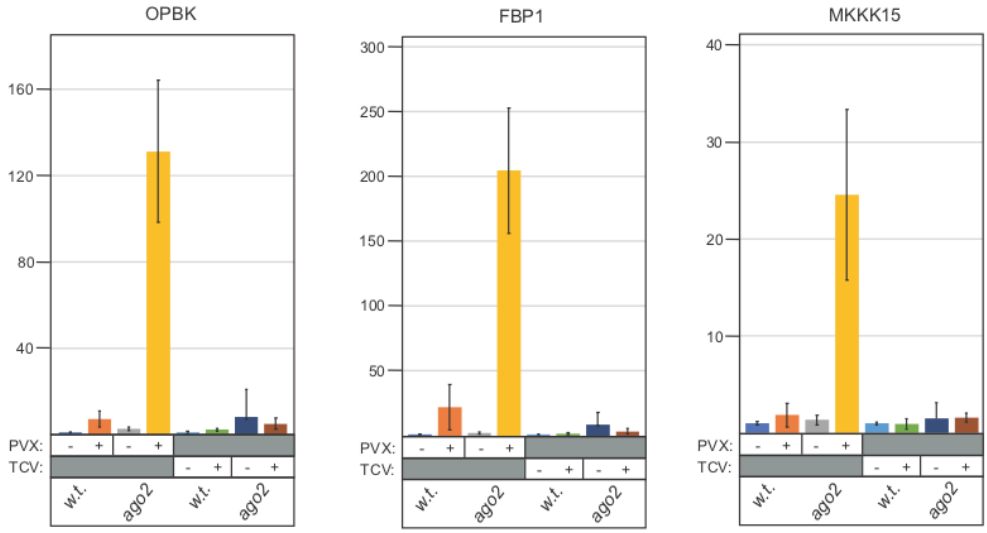

Figure 2. Analyses of the expression of the *OPBK*, *FBP1* and *MKKK15* genes in virus infected *N. benthamiana* plants.

Homozygote glucan endo-1,3-beta-glucosidase (*GE1,3βG*) mutant plants have already been obtained and their sensitivity to virus infection tested. The *ge1,3βg* mutants exhibited increased sensitivity to both PVX and TCV infection, confirming the gene's involvement in general antiviral stress responses. The inactivation of WRKY transcription factor 6 (WRKY6) has also been attempted. After repeated tries however, only heterozygote *WRKY6* plants could be obtained, indicating that the homozygous *wrky6* mutants are embryonic lethal. From group 2, we chose the *OPBK* and *FBP1* to inactivate their genes. We currently have heterozygous plants for both genes. Establishment of homozygote mutants are under way. Further analyses of these mutants, will help us to elucidate the role of the *OPBK* and *FBP1* genes in PVX-induced systemic necrosis.

9. Identification of functionally important regions of AGO2.

Using multiple sequence alignments of AGO proteins from evolutionarily distant species, we have identified several conserved amino acid residues of *N. benthamiana* AGO2, which might serve as sites for post-translational modifications. These are the following:

1. PS846-847 in the PIWI domain

2. Y281 in the N domain

3. S1028 at the C terminus.

We have mutated all of these sites using site directed mutagenesis. Several mutants were created in the PS846-847 motif: S847A, S847D and PS846-847AA. In transient reporter assays both the S846A and S847D mutants retained their ability to silence gene expression, although to a somewhat reduced degree than wild-type AGO2. The double mutant PS846-847AA was unable to silence gene expression. Rather, it functioned as a dominant negative molecule, similarly to the D907A catalytic center mutant. Surprisingly, in substrate cleavage assays all three mutants proved to be inactive. These results provide direct proof that cleavage activity and the ability to inhibit translation can be uncoupled in plant AGOs, similarly to that observed in animals. (Fig. 3).

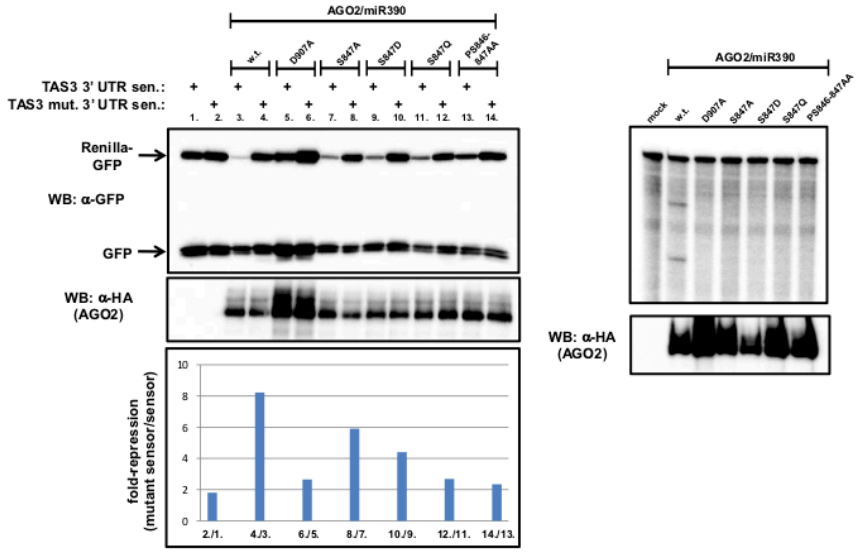

Figure 3. Functional analyses of AGO2 mutants in transient reporter assays (left) and substrate cleavage assays (right)

The Y281 residue is located in a highly conserved region of the N domain, and was also identified as a phosphor acceptor site in *Arabidopsis* AGOs in earlier high throughput studies. Nonetheless, no distinct function has been assigned to this region of the molecule yet. We created a Y281F mutant of AGO2 and tested its ability to inhibit translation in transient reporter assay. Interestingly, the mutation did not reduce the ability of AGO2 to inhibit translation at all (data not shown). We also created the equivalent mutant of *N. benthamiana* AGO1 (Y218F). This molecule was completely inactive in substrate cleavage assay. In light of these results, it will be interesting to test the cleavage activity of AGO2(Y281F) as well. The S1028 residue is also highly conserved in AGO molecules. The S1028A mutation did not influence the ability

of AGO2 to inhibit gene expression (data not shown). Further studies will be carried out to confirm the post-translational modification and functional role of these evolutionarily conserved residues of AGO2.

10. Establishment of ago2 mutant tomato lines.

Tomato production is highly affected by viral diseases that are responsible for millions of dollars in production losses. Many viruses infecting tomato have been described, while new viral diseases keep emerging. Pepino Mosaic Virus (PepMV) is a rapidly spreading virus, which has established itself as one of the most important viral diseases in tomato production over recent years. Recent results have indicated that the expression of *AGO2* is increased in PepMV infected tomato plant raising the possibility that *AGO2* contributes to anti-PepMV defense in this plant species. To directly examine the role of *AGO2* during PepMV infection, we initiated the inactivation of the *AGO2* gene of tomato. Using CRISPR/Cas9 genome editing we successfully established several *ago2* mutant tomato plant lines (Fig. 5).

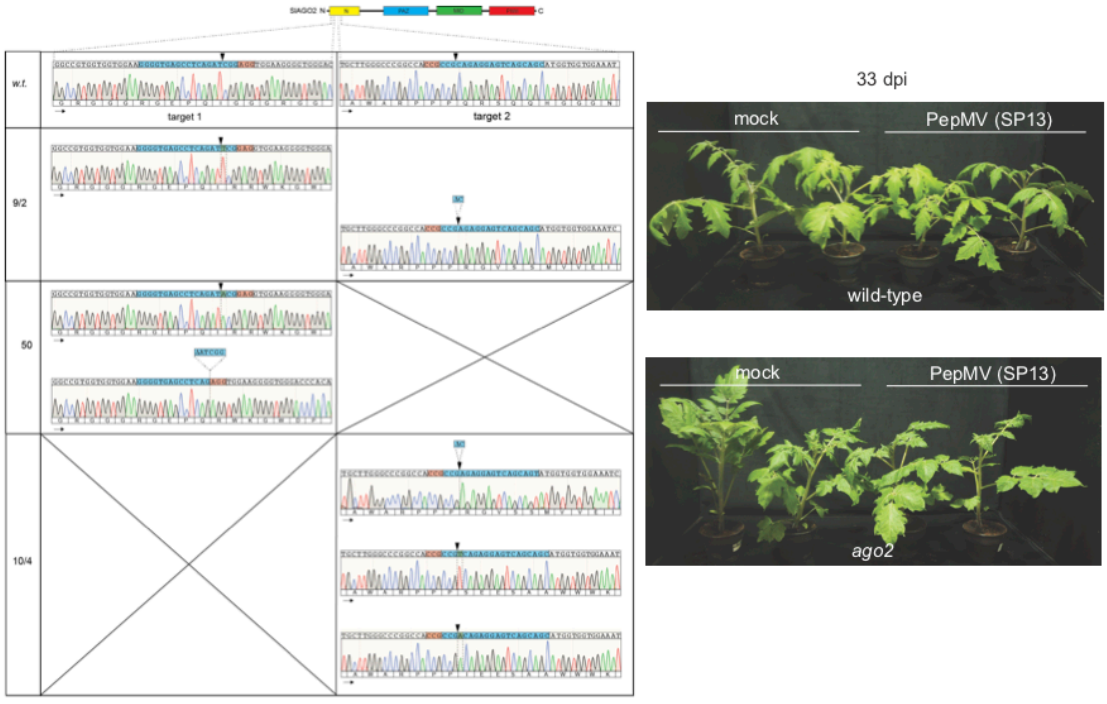

Figure 5. Establishment of *ago2* mutant tomato lines. Two regions in the N domain of *AGO2* were targeted (left). Testing of the PepMV sensitivity of *ago2* mutant tomato plants (right)

The *ago2* mutant tomatoes exhibited mild alterations in leaf morphology compared to wild-type plants. Apart from that however, no significant phenotypic differences could be noted between the mutant and wild-type plants. The sensitivity of the *ago2* tomato plants towards PepMV infection was also tested. The SP13 isolate of PepMV was used to infect the mutant

plants. As control, parental Money Maker tomato plants were also infected with the virus. At 14 dpi PepMV RNA level was monitored in the infected plants. No statistically significant difference in the accumulation of viral RNA could be noted between the two cohorts of plants (data not shown). Consistently, the symptoms of infection exhibited by the *ago2* mutant and wild-type plants were essentially identical and generally very mild. In the future, we plan to test more aggressive PepMV isolates and other pathogenic viruses on our mutant tomatoes, to further examine the contribution of *AGO2* to antiviral defense in this plant species.

11. Uncovering the antiviral role of AGO2 and RDR6 in PepMV infected N. benthamiana plants.

Earlier, *N. benthamiana* has been employed successfully as an experimental host for PepMV. Thus, as a surrogate for tomato, we decided to use *N. benthamiana*. Susceptibility of our *N. benthamiana* mutants to PepMV infection was tested. We determined that *ago2* mutant *N. benthaniana* exhibits increased sensitivity to PepMV infection (Fig. 6). Contrary, disabling of *AGO1A*, *AGO1B* or *AGO5* had no significant effect on the progression of the disease (data not shown). Surprisingly, *ago10* mutant *N. benthamiana* exhibited faster recovery from PepMV infection than the wild-type plants. This finding indicates that *AGO10*, unlike *AGO2*, plays a pro-viral role during PepMV infection. In addition, using *rdr6* mutant and *ago2/rdr6* double mutant *N. benthamiana* plants, we were able to establish that most of the viral siRNAs produced during PepMV infection are *RDR6*-dependent secondary siRNAs. The manuscript, which reports the above findings, is currently under preparation.

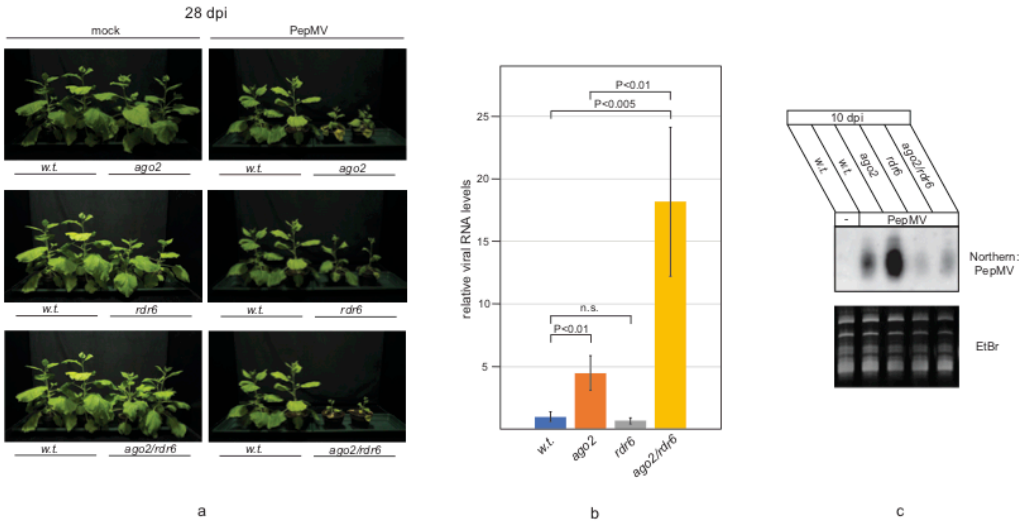

Figure 6. *AGO2* and *RDR6* play important roles in PepMV infection in *N. benthamiana*. PepMV infected *ago2* plants exhibit more severe symptoms (a) and accumulate significantly higher levels of virus than wild-type plants (b). Majority of PepMV derived vsiRNAs are *RDR6*-dependent secondary vsiRNAs (c).

SIGNIFICANT DEVIATIONS FROM THE PROPOSED RESEARCH PLAN

Analyses of AGO2-containing protein complexes

In the original Research plan establishment of *N. benthamiana* lines stably over-expressing epitope tagged forms of wild-type and mutant forms of AGO2 proteins was proposed. We planned to use these plants for the purification of AGO2 containing complexes with the aim of identifying novel, AGO2 associated proteins. The establishment of these plant lines however, took longer than expected, as most of the obtained lines expressed the tagged AGO2 proteins at very low levels. The underlying cause of this problem is not completely understood. Eventually however, we were able to successfully establish several high-expressor plant lines with most of the AGO2 constructs. Because of the technical difficulties mentioned above, this objective of the project could not be completed in the time frame of the K124705 grant. Nonetheless, because of the potentially substantial scientific value of studying the composition of AGO2 protein complexes, we intend to carry out these analyses in the near future, relying on the plant lines that are generated during the time period covered by this grant.

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