Final report of NK-105850 project

Summary
The goal of the this study to better understand the RNA silencing based antiviral mechanism and discover the multiple role of RNA silencing in the plant–virus interaction. We have used several tools of molecular biology including transgenic plants, genome editing (CRISPR/CAS9), highthroughput technologies (such as deep sequencing, analysis of RNA targeting and gene expression at genome wide level, etc.) and bioinformatics to explore in more details of the intimate interaction between the invading virus and the plant cell.
We explored the variable role of plant dicers (Dicer like proteins, DCLs) in the biogenesis of antiviral viral siRNAs in particularly the origin and generation of Argonaute (AGO) competent vsiRNAs.
Similarly we were able to identify those AGO proteins which play inportant role in antiviral response in distinc plant-virus system.
The specific effects of the potent viral encoded p19 silencing suppressor protein on host small RNAs and theirs target transcripts were also identified.

Background
Viruses are among the most important plant pathogens that cause huge economic losses in many agriculturally important crops worldwide. The invasion of the host by viruses deeply alters the physiology of the plants at cellular and tissue levels due to the interaction of the virus with the cellular pathways, which ultimately leads to viral symptom development. During evolution, plants have developed diverse strategies to combat virus infections. Amongst others, RNA silencing is one of the most important mechanisms that serve to fight against viruses.
RNA silencing is a conserved eukaryotic pathway involved in almost all cellular processes like development, stress responses and antiviral defense. RNA silencing relays on the 21-24 nt short interfering RNAs (siRNAs) or micro RNAs (miRNAs) the hallmark molecules of silencing. The siRNAs and the miRNAs (collectively named small RNAs, sRNAs) are processed by RNase III-type ribonucleases, the DICER (in plants Dicer-Like, DCL) enzymes in collaboration with their partner DOUBLE-STRANDED RNA BINDING (DRB) proteins. sRNAs then associate with ARGONAUTE (AGO) proteins the catalytic core of RNA Induced Silencing Complex (RISC) and guide RISC to silence cognate RNAs through cleavage or translational repression (post-transcriptional gene silencing, PTGS) or induce chromatin/DNA modifications of the specific genomic locus (transcriptional gene silencing, TGS). In some specific cases, amplification of silencing occurs through double-stranded RNA synthesis by RNA-dependent RNA polymerases (RDRs) and secondary siRNA production. sRNAs are non-cell autonomous, they can move within the plant to transmit gene silencing from cell-to-cell or systemically on long distance as mobile silencing signals.

Results
DCL2 and DCL4 are equally important in the antiviral response in virus *Nicotiana benthamina*

We have analyzed the viral siRNA biogenesis process and our results suggest that the previously defined hierarchical operation of DCL2 and DCL4 plant Dicers do not operate in the same mode in all plant-virus interaction. In Arabidopsis plant the primary antiviral Dicer is the 21 nt siRNA-generating DCL4, and if it does not work, it is replaced by the 22nt siRNA generating DCL2. In contrast, our results strongly suggest that in *Nicotiana benthamiana* infected by Cymbidium ringspot virus (CymRSV) both DCL2 and DCL4 are equally important in the plant antiviral response (Figure 1). These results suggests that the role of plant dicers likely varies in different plat and virus combination (Kontra et al., manuscript in preparation).

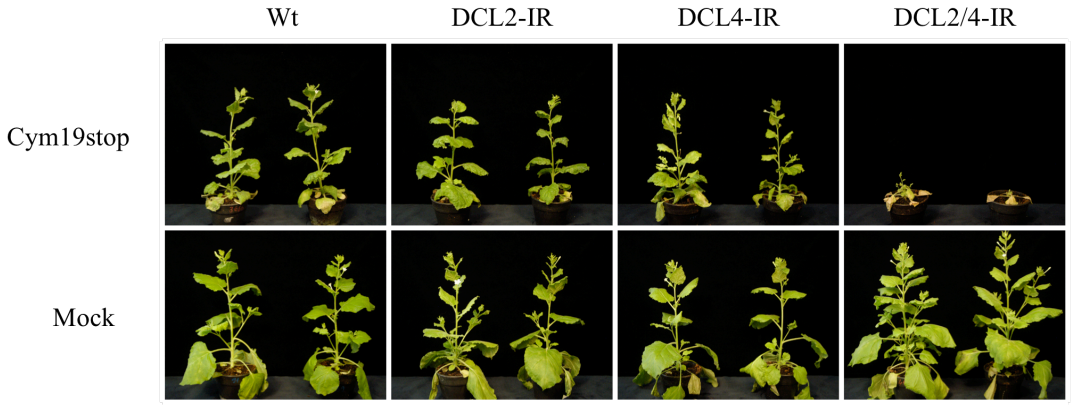


Figure 1. Wild type (wt) N. benthamina and DCL2 (DCL2-IR), DCL4 (DCL4-IR) and DCL2 and DCL4 (DCL2/4-IR) silenced plants were infected by silencing suppressor defective mutant (Cym19stop) of CymRSV. Both DCL-IR and DCL4-IR plants were able to recover from virus infection, indicating the effective antiviral response. In contrast, DCL2/4 silenced plant did not recover from virus infection, demonstrating that both DCL2 and DCL4 are equally required from the effective antiviral silencing.

Distinct Argonaute proteins have inportant function in the antiviral response in different virus-host interactions

The role AGO proteins in plant virus interaction was mostly studies in Arabidopsis plants, however the antiviral role of AGO proteins in other plants are poorly explored. Indeed, the AGO proteins in the virological model plant (*Nicotiana benthamiana*) are poorly analysed. In particularly, the role of AGO2 has been obscure until recently. Recent data, however, have indicated its involvement in various biotic and abiotic stress responses. Despite its suggested importance, there is no detailed characterization of this protein to date. Therefore, we cloned and characterized at molecular level of the AGO2 protein of *N. benthamiana.* To monitor the activity of AGO2, we have established a sensitive transient *in vivo* reporter system. We find that in various miRNA target site constellations (ORF, 3'UTR) AGO2 is capable of exerting translational repression, most probably by invoking different mechanisms. Our data also show that an intact catalytic center is a prerequisite for efficient translational repression by AGO2. However, this is most likely necessary for RISC maturation rather than target cleavage *per se*. Mismatches between the 3' end of the miRNA guide strand and the 5' end of the target site enhanced gene silencing by AGO2, a feature not exclusive to this Argonaute, as AGO1 displayed similar target preferences. Additionally, based on structural and bioinformatics considerations several amino acid residues of *N. benthamiana* AGO2 have been identified that were expected to affect various aspects of the protein's function. Mutation

of these amino acids confirmed most of the predictions, revealing profound similarities between the RISC activation mechanisms of plants and animals.

The results of this part of our project was published in:
Functional dissection of a plant Argonaute.
Fátyol K, Ludman M, **Burgyán J**.
Nucleic Acids Res. 2016 Feb 18;44(3):1384-97

Antiviral role of the AGO2

To examine the antiviral role of the AGO2 protein of *N. benthamiana* plant, we decided to generate *ago2* mutant plants using the type II CRISPR/Cas9 system of *S. pyogenes*.
We created a single plasmid system to express the Cas9 protein and the appropriate sgRNA. The sgRNA, targeting the functionally essential PIWI domain of the expressed *AGO2* gene was designed and generated. The CCTop plant CRISPR analysis online tool did not detect off target sites for this sgRNA in coding regions of *N. benthamiana*.
The pK7WG2D binary plasmid vector was modified to co-express the sgRNA and the plant codon optimized SpCas9 nuclease. The efficiency of the generated sgRNA was confirmed by transient assay system. *Agrobacteria* carrying the binary targeting construct was infiltrated into *N. benthamiana* leaves. Genomic DNA was extracted from the infiltrated leaf tissue and the targeted region of the *AGO2* gene was PCR amplified and sequenced. We detected Cas9 introduced mutations at the expected position in 6,25% of the analyzed DNA fragments. Based on this results stably transformed *N. benthamiana* plants were generated using the established sgRNA.
Mutations in the *AGO2* gene were detected at the expected position in all transformants (Figure 2). Three of the examined plants carried wild-type *AGO2* alleles, in addition to the mutant ones, indicating that the parental T0 plant was indeed chimeric. Importantly however, in lines #1.3 and #1.4 only two of the above-described *ago2* mutant alleles were detected. These biallelic plants were again allowed to self-pollinate and the resulting T2 progenies were used in all subsequent experiments as *ago2* mutant plants. The phenotype of these plants was indistinguishable from the parental wild-type *N. benthamiana* plants, under the growth conditions used in our experiments.

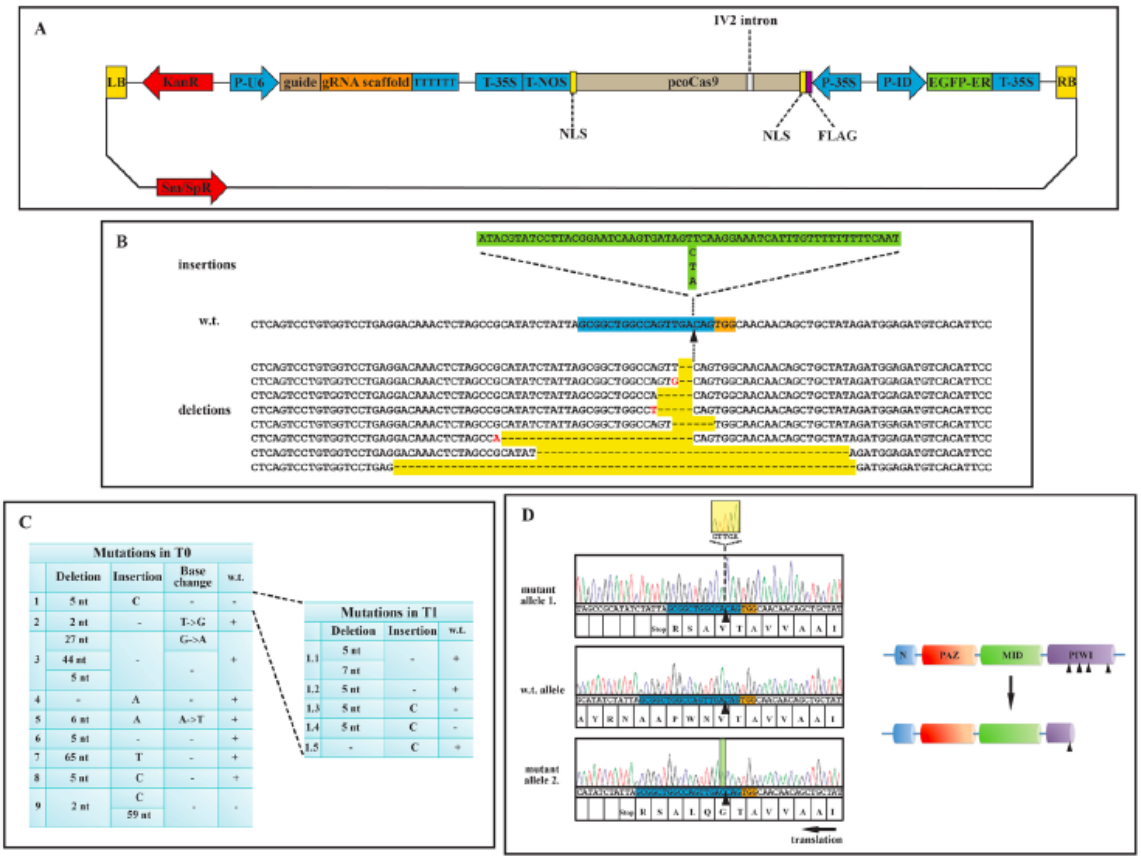


Figure 2. Generation of *ago2* mutant *N. benthamiana*. (**A**) Schematic structure of the bi-functional Cas9-sgRNA expression plasmid used for the targeted inactivation of the *AGO2* gene of *N. benthamiana*. (**B**) Structure of the isolated *ago2* alleles. The targeted sequence of the wild-type *AGO2* gene is highlighted with blue and the adjacent PAM motif with orange. In the isolated mutant alleles the insertions are highlighted with green and the deletions are with yellow. The nucleotide changes accompanying some of the deletions are indicated by red letters. (**C**) Summary of the mutations identified in the T0 and T1 transformant plants. (**D**) The mutations in the *ago2* alleles of transformant lines 1.3 and 1.4 result in premature stop codons. Both alleles encode truncated, dysfunctional AGO2 proteins, which lack three out of the four catalytically essential amino acids of the PIWI domain.

Using the generated *ago2* mutants of *N. benthamiana* we demonstrated that AGO2 had differential effects on the plants' antiviral responses, upon which the viruses could be categorized into two groups:

(1) AGO2 was a critical component of the antiviral response. The viruses that belonged to this group (*Potato virus X*, PVX; *Turnip mosaic virus*, TuMV and *Turnip crinkle virus*, TCV) were quite dissimilar and the symptoms they elicited were also diverse. The lack of AGO2 resulted in apical necrosis and eventual lethality in both PVX and TuMV infected plants. Contrary, during TCV infection no localized necrosis was evident, however after 4 weeks general exacerbation of symptoms resulted in the death of the *ago2* plants.

(2) AGO2 had no or only a minor, modulatory effect on virus infection. This group included various tombusviruses and *Cucumber mosaic virus*, CMV. The *AGO2* status of the plants did not influence the infection process caused by wild-type tombusviruses and only slightly affected the rate of recovery from infections elicited by their silencing suppressor protein deficient forms. These results are in agreement with the recent results suggesting that AGO1 is the main antiviral AGO involved in tombusvirus infection (see

the next paragraph). The symptoms of CMV infection on wild-type and *ago2* mutant *N. benthamiana* plants were essentially indistinguishable.

These results were published in our recent publication:
Crispr/Cas9 Mediated Inactivation of Argonaute 2 Reveals its Differential Involvement in Antiviral Responses.
Ludman M, **Burgyán J**, Fátyol K.
Sci Rep. 2017 Apr 21;7(1):1010.

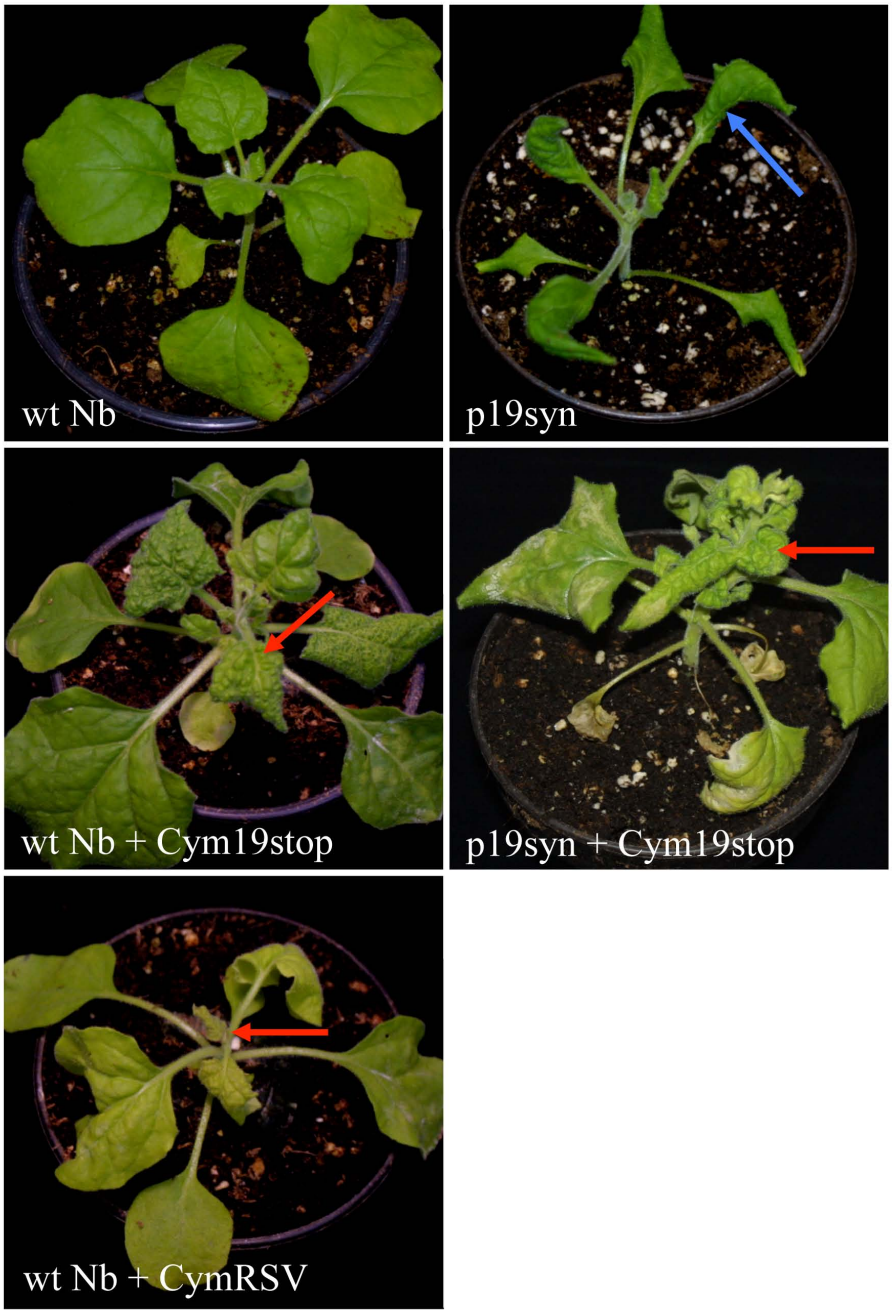
Distinct Effects of p19 RNA Silencing Suppressor on Small RNA Mediated Pathways in Plants

Viruses, to counteract host defense, have evolved viral suppressors of RNA silencing (VSRs) providing strong evidence for the antiviral nature of silencing. Most viruses studied so far were found to encode at least one VSR. VSRs were shown to block silencing at multiple steps like initiation, effector complex assembly, silencing amplification but also through transcriptional control of endogenous factors, hormone signal modulation or interaction with protein-based immunity. The absence or inactivation of VSRs leads to the recovery of plants from viral infections, demonstrating the effect of plant antiviral silencing response. Although several VSRs have been identified in the past, our knowledge about the precise molecular basis of their action and theirs multifunctional roles have only been resolved in a few cases. The p19 protein of tombusviruses is one of the best-known VSR. Our previous crystallographic studies have shown that p19 tail-to-tail homodimer acts as a molecular caliper to size-select and sequester siRNA duplexes in a sequence-independent manner preventing the loading of siRNAs into AGO effector proteins.

Since the endogenous and antiviral functions of RNA silencing pathway rely on common components, it was suggested that viral suppressors interfere with endogenous silencing pathway contributing to viral symptom development. Therefore we aimed to understand the effects of the tombusviral p19 suppressor on endogenous and antiviral silencing during genuine virus infection.

To better understand the specific effect of p19 VSR on antiviral silencing and endogenoussmall RNA pathways, we generated a *N. benthamiana* plant (p19syn) capable of sustaining the ectopic expression of the *Cymbidium ringspot virus* (CymRSV) p19 upon infection with a suppressor-deficient CymRSV (Cym19stop; Figure 3).

Figure 3. Comparison of p19syn plants' phenotype with CymRSV- or Cym19stop-infectedviral symptoms in wt or p19syn plants. Characteristic phenotype of p19syn (1–57 line)(blue arrow) and virus-induced systemic symptoms on wt or p19syn plants (red arrow) are shown. Pictures were taken at 10 dpi.



By using wt and p19syn plants in combination with CymRSV and Cym19stop, we were able to analyze the effects of p19 provided "in trans" and "in cis" during the viral invasion of the plant. We have shown that p19 can efficiently sequester endogenous small RNAs (sRNAs) in mock-inoculated p19syn plants while it does not bind these sRNAs upon Cym19stop infection. Also, the presence of p19 in virus infection did not alter the expression of miRNAs significantly (Figure 4).

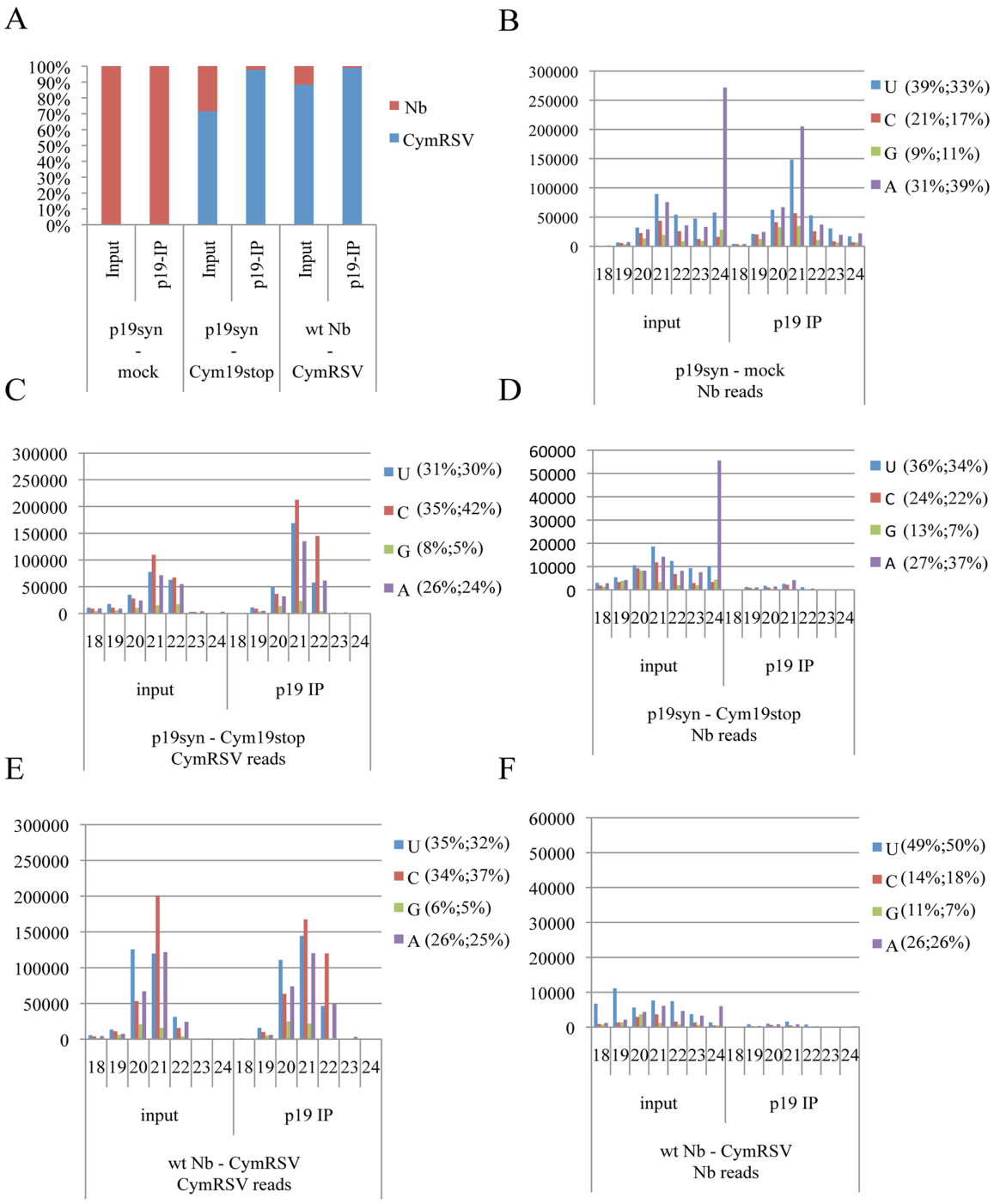


Figure 4. Endogenous and viral sRNAs sequestration preferences of p19. (A) Total and p19-bound vsiRNAs and endogenous sRNAs derived from *N. benthamiana* and p19syn plants infected with wt (CymRSV) and suppressor deficient Cym19stop viruses and mock-inoculated plants. (B) input and p19-bound endogenous sRNA duplexes in mock-inoculated plants. (C-F) inputs and p19-bound vsiRNAs and endogenous sRNAs when p19 was expressed in *trans* (C and D) or *in cis* (E and F) during virus infections as indicated. The size classes of sRNAs between 18 and 24nt are indicated by numbers The 5' nucleotides are indicated by color codes. The percentages of specific 5'-nucleotide sRNAs in input and p19 IP are shown in brackets at the right side of the panel (input%; IP%). Note that B, C, E scales differ from D and F ones. Read counts were normalized to 1 million reads.

These findings do not support the widely accepted assumption that viral symptoms are the direct consequence of the impact of VSRs on endogenous silencing pathways.

To better understand the biological relevance of vsiRNA-mediated endogenous sRNA binding and out-competition/release from p19 sequestration we analyzed the behavior of known miRNA-target mRNA pairs. We compared RNAseq data obtained from mock-inoculated p19syn plant samples (when p19 binds to miRNAs) and from Cym19stop virus-infected p19syn plant samples (when p19 binds preferentially vsiRNAs while miRNAs are outcompeted/released). In the absence of the virus, p19 efficiently bound miRNA duplexes and this correlated with elevation of most of the miRNA-target mRNAs as the consequence of miRNA duplex sequestration by p19 and inability to program miRISC for cleavage (p19syn compared to wt *N. benthamiana*). Upon Cym19stop virus infection however, the levels of most miRNA target RNAs were downregulated (compared to mock-infected p19syn) as the consequence of miRNA out-competition/release from p19 (Figure 5).

Finally using AGO1- and AGO2- immunoprecipitation experiments we observed that p19 specifically compromises vsiRNAs' loading into AGO1 but not AGO2. Since antiviral silencing is strongly inhibited by p19, this suggests that AGO1 is the main effector protein against CymRSV tombusvirus (Figure 6).

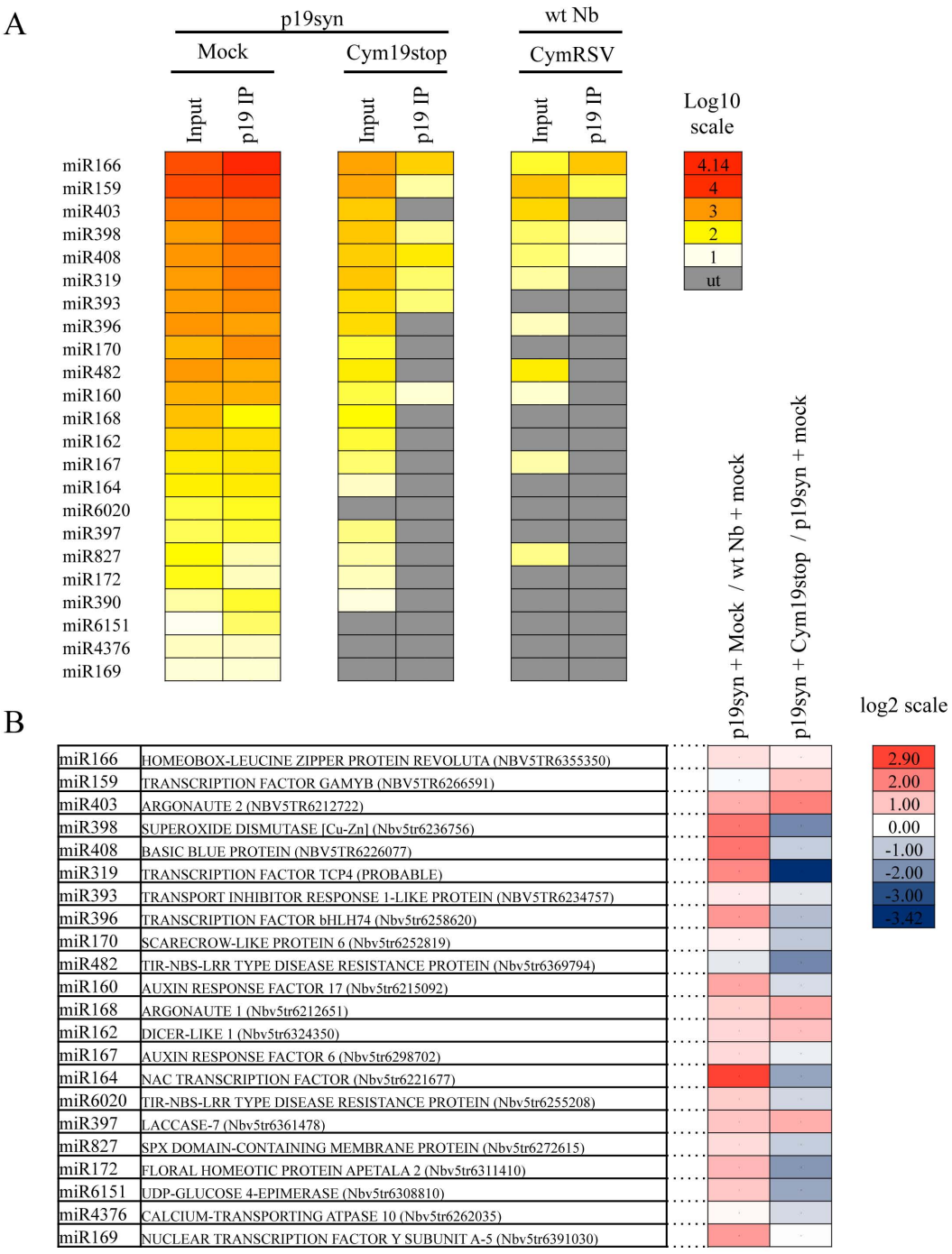


Figure 5. (A) Abundance of p19-bound miRNAs (listed on the left) in mock- or Cym19stop-infected p19syn plants and in CymRSV-infected wild type plants. The normalized total reads are shown on a log10 scale, values under threshold (ut). Heatmap legend is shown on the right. Read counts were normalized to 10^6 *N. benthamiana* genome matching read counts. (B) Ratio of miRNA-targeted mRNAs in mock-infected p19syn plants (relative to mock-infected wt *N.benthamiana*) and Cym19stop-infected p19syn plants (relative to mock-infected p19syn plants). Heatmap legend is shown on the right on a log2 scale.

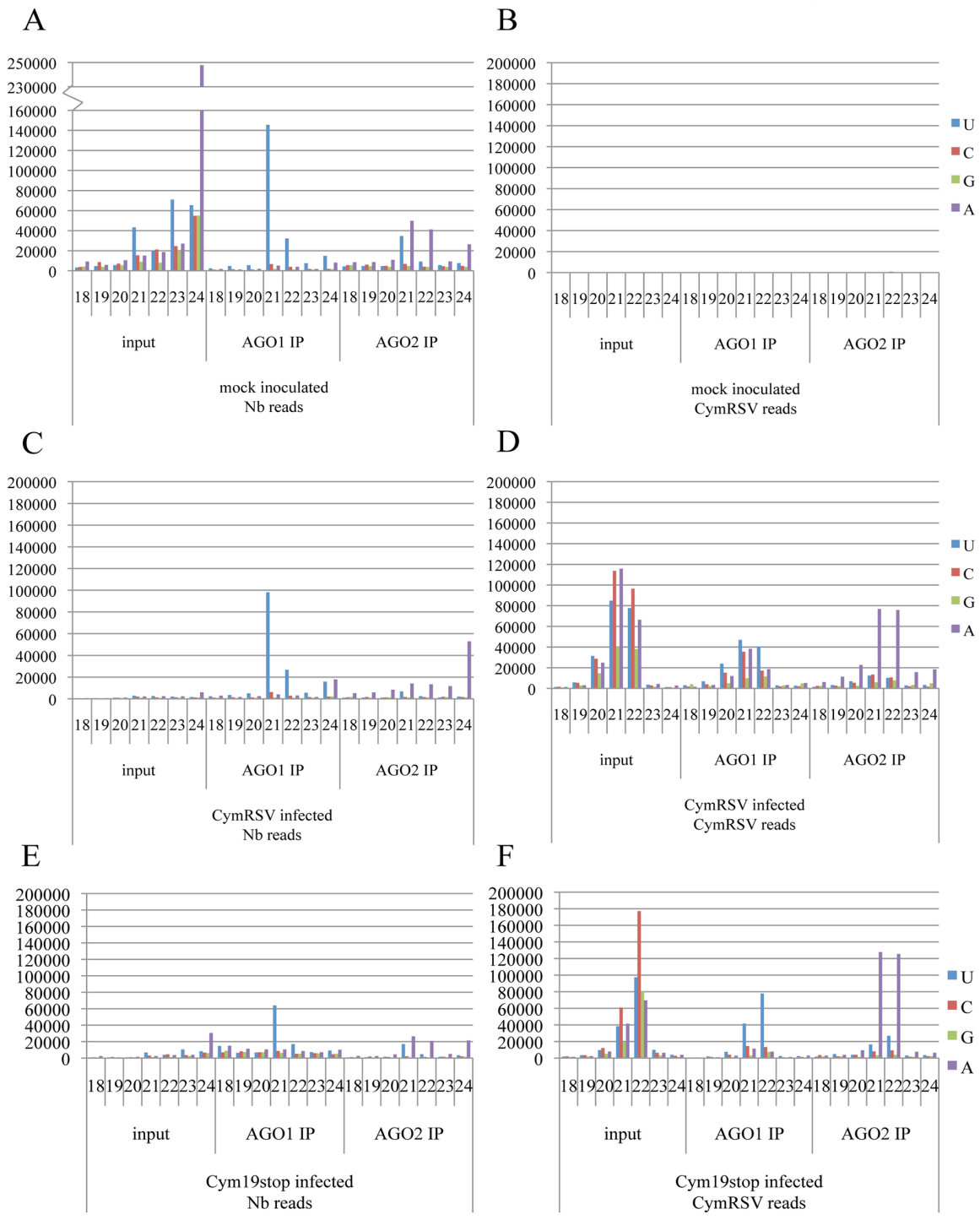


Figure 6. Deep sequencing analysis of AGO1- and AGO2-bound sRNAs. *N. benthamiana* specific reads from mock-inoculated (A), CymRSV- (C) and Cym19stop-infected wild-type plants (E). The vsiRNA reads of the same samples are presented in (B), (D) and (F) respectively. 5' nucleotides of vsiRNAs are indicated by color code on the right. Size classes (nucleotide) of sRNAs are indicated by numbers.

The obtained results demonstrated that AGO1 has a major role in antiviral response against tombusvirus infection. However, the additional role of other plant AGOs in

antiviral response remained to be explored and it likely depends on specific features of the highly diverse plant viruses. The availability of CRISPR/ Cas9 system for plant research will also help to clarify the specific roles of plant effectors in
antiviral silencing response.

These results were published in our recent publication:

Distinct Effects of p19 RNA Silencing Suppressor on Small RNA Mediated Pathways in Plants.

Kontra L, Csorba T, Tavazza M, Lucioli A, Tavazza R, Moxon S, Tisza V, Medzihradszky A, Turina M, **Burgyán J.**

PLoS Pathog. 2016 Oct 6;12(10):e1005935.