Functional dissection of a plant Argonaute

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ABSTRACT

RNA guided ribonuclease complexes play central role in RNA interference. Members of the evolutionarily conserved Argonaute protein family form the catalytic cores of these complexes. Unlike a number of other plant Argonautes, the role of AGO2 has been obscure until recently. Newer 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. Here we report cloning and molecular characterization of the AGO2 protein of the virological model plant *Nicotiana benthamiana*. We show that AGO2 can directly repress translation via various miRNA target site constellations (ORF, 3′ UTR). Interestingly, although AGO2 seems to be able to silence gene expression in a slicing independent fashion, its catalytic activity is still a prerequisite for efficient translational repression. Additionally, mismatches between the 3′ end of the miRNA guide strand and the 5′ end of the target site enhance gene silencing by AGO2. Several functionally important amino acid residues of AGO2 have been identified that affect its small RNA loading, cleavage activity, translational repression potential and antiviral activity. The data presented here help us to understand how AGO2 aids plants to deal with stress.

INTRODUCTION

RNA silencing (or RNA interference, RNAi) is an evolutionarily conserved gene regulatory mechanism with fundamental implications in many biological processes (1). It is triggered by double-stranded RNA (dsRNA) and results in the repression of genes containing sequences identical or highly similar to the initiating dsRNA. In plants, RNA silencing acts at both the RNA and DNA levels (2,3). Mechanisms of silencing at the RNA level include mRNA cleavage or translational repression (post-transcriptional gene silencing, PTGS), whereas at the DNA level they in-

volve DNA and/or histone methylation and subsequent transcriptional gene silencing (TGS). All these manifestations of RNA silencing rely on the action of small RNA (sRNA) molecules of 21–24 nucleotide (nt) that derive from the processing of the dsRNA trigger by RNaseIII-like enzymes called Dicer, or Dicer-like (DCL) in plants. Upon processing, one strand of the sRNA duplexes is incorporated into RNA-induced silencing complexes (RISCs) to guide sequence-specific inactivation of the targeted RNA or DNA.

RNA silencing also constitutes the primary plant immune system against viruses (4–6). Antiviral RNA silencing is triggered by dsRNA replication intermediates or intramolecular fold-back structures within viral genomes. These viral dsRNAs are processed by DCLs to produce viral small interfering RNAs (vsiRNAs), which are subsequently incorporated into antiviral RISCs. Many viruses have evolved various means to evade detection and neutralization by the above mechanisms. For instance, they deploy proteins—viral suppressors of RNA silencing (VSRs)—that interfere with RNA silencing at different steps.

A common denominator of all of the above processes is that Argonaute (AGO) proteins play a central role in their final executive stage. Members of this evolutionarily conserved protein family form the catalytic core of RISC (7). In plants, Argonautes can be divided into three major phylogenetic clades (8,9). Members of the first clade (AGO1/AGO5/AGO10 in *Arabidopsis*) primarily bind 21-nt small RNAs (most miRNAs and 21-nt siRNAs) and are the main executors of PTGS. The 24-nt siRNAs that derive mostly from transposons and repetitive sequences are incorporated into AGOs that belong to clade 2 (AGO4/AGO6/AGO9 in *Arabidopsis*,). They silence transcription of the genomic loci, from which the 24-nt siRNAs originate. Clade 3 includes *Arabidopsis* AGO2, AGO3 and AGO7. AGO7 is a highly specialized Argonaute. It regulates the auxin-signaling pathway via production of *trans*-acting siRNAs (tasiRNAs). The *Arabidopsis* AGO2 and AGO3 genes are very similar to each other and are likely the result of an evolutionarily recent duplication event. For a long time, no active regulatory role was assigned to AGO2.

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Rather, it was proposed that it could prevent miRNA action, because redirection of some miRNAs into AGO2 seemed to halt them from fulfilling their normal biological activity (10). In the last 4 years, however, a number of papers have been published indicating the essential role of AGO2 in a variety of processes, mostly related to the ways plants deal with stress:

(1) AGO2 accumulation is induced by viruses and it participates in plant antiviral responses. In some cases this takes place in cooperation with other Argonautes (AGO1 or AGO5) while in others, AGO2 can independently accomplish viral restriction (11–15). The antiviral activity of AGO2 is thought to rely predominantly on its ability to slice viral RNAs; however, repression of translation of viral proteins can be an important mode of action as well.

(2) Viral infection triggers the production of an abundant class of endogenous siRNAs. These virus-activated siRNAs (vasiRNAs) target more than 1000 host genes for silencing in an AGO2-dependent manner and suggested to confer broad-spectrum antiviral activity in addition to the virus-specific antiviral response (16).

(3) AGO2 also provides antibacterial resistance. It is highly induced by the bacterial pathogen *Pseudomonas syringae*. By binding miR393b*, AGO2 inhibits the expression of the SNARE gene MEMB12, thereby increasing the exocytosis of antimicrobial PR proteins (17).

(4) Recent studies have reported the production of small RNA species from near the vicinity of DNA double strand breaks (DSB) in a variety of organisms, including plants (18). The DSB-induced small RNAs (diRNAs) are recruited by AGO2, which is the only Argonaute induced by DNA damage. The AGO2/diRNA effector complexes are hypothesized to contribute to the repair of DSBs by serving as scaffolds for the assembly of repair foci. In addition, AGO2 may protect the cell from the toxic effects of truncated proteins by inhibiting the translation of nascent aberrant transcripts originating from the damaged loci.

The only plant Argonaute, which has been studied more thoroughly so far, is AGO1 (19). The above examples, however, clearly emphasize the necessity to understand the mode of actions of plant AGO2 in its molecular details as well. Thus, we set out to clone and characterize the AGO2 protein of the virological model plant *Nicotiana 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. In summary, the results we report here will help us to understand how AGO2—a previously poorly characterized RNA silencing executor—aids the adaption of plants to various biotic and abiotic stresses.

MATERIALS AND METHODS

Plasmid construction

Plasmids were generated using standard techniques (20). Sub-fragments of *N. benthamiana* AGO ORFs were amplified from cDNAs prepared from leaves using polymerase chain reaction (PCR). The full-length AGO ORFs were assembled in pGEM-T easy plasmid vector and subsequently cloned into pBIN61 binary vector. Tandem FLAG-HA epitope tags were added to the N-termini of the proteins using oligonucleotides. To generate sensor plasmids, the Renilla luciferase ORF was cloned into pENTR11 plasmid. Next, oligonucleotides corresponding to wild-type or mutant miRNA binding sites were inserted into the pENTR11-Renilla vector. Finally, the Renilla luciferase expression cassettes (along with the miRNA binding sites) were transferred into the pK7WGF2 binary vector in frame with the GFP ORF using LR clonase. The *N. benthamiana* miR390B, miR168A and *A. thaliana* miR408 genes were amplified by PCR and cloned into pENTR11 plasmid vector. The miRNA ORFs were then transferred into pK7WG2D binary vector using LR clonase. Site directed mutagenesis of AGO2 was performed with specific mutagenesis primers using Pfu or Phusison polymerases. Sequences of all plasmid vectors were verified by sequencing.

Agroinfiltration, protein analysis, reporter assays

N. benthamiana leaves were infiltrated with suspensions of *Agrobacteria* carrying the binary expression vectors and sensors as previously described (21). Before infiltration the *Agrobacteria* strains were diluted to $OD_{600} = 1$. Protein expression was analyzed three days post-infiltration.

Infiltrated leaves were frozen in liquid N_2, milled and extracted with a lysis buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 5 mM NaF, 1 mM dithiothreitol, 0.5 mM Na_3VO_4. The lysis buffer was supplemented with 1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor mix (Roche Molecular Biochemicals) before use. The proteins were resolved on 8% polyacrylamide gels and transferred to PVDF membrane (Amersham Pharmacia Biotech). Transfer and subsequent processing of the membranes were performed according to the manufacturer's instructions. The following antibodies were used: anti-GFP (Sigma-Aldrich), anti-HA (3F10) (Roche), anti-FLAG (M2) (Sigma-Aldrich), anti-actin (plant) (Sigma-Aldrich). For detection, the Clarity Western ECL substrate was used from Bio-Rad. Western blots were imaged on a Bio-Rad ChemiDoc XRS Plus System and quantified with Image Lab software.

For the reporter assays *N. benthamiana* leaves were infiltrated with suspensions of *Agrobacteria* as indicated. On the left side of the leaf the wild-type sensor, while on the right side of the same leaf the mutant sensor containing *Agrobacteria* suspensions were infiltrated. Three leaves per plant were infiltrated with the same suspensions. Protein expressions in the infiltrated leaves were monitored three days post-infiltration. Discs (50 mg each)—cut from leaves infiltrated with the same *Agrobacteria* suspensions—were pooled and protein lysates were prepared as described above. The lysates were analyzed by western blot. All reporter assays were repeated at least three times and representative experiments are shown.

Analysis of antiviral activity of AGO2

Agrobacteria strains carrying wild-type or mutants of AGO2 were infiltrated into the right side of *N. benthamiana* leaves along with the PVX-GFP-ΔTGB strain. Into the left side of the same leaves PVX-GFP-ΔTGB was infiltrated with 'empty' vector (pBIN61) containing *Agrobacteria*. Three days post-infiltration total protein extract and RNA were prepared from the two sides of the leaves. Leaf tissues were pooled from three leaves infiltrated with the same *Agrobacteria* suspensions. The virally encoded GFP protein expression was analyzed by western blot. The viral RNA was detected by northern hybridization using a GFP-specific probe. Amplification of viruses was compared between the left and right side of the same leaf.

RNA analysis

Total RNA extraction from leaf tissue and subsequent RNA blot analysis of high molecular weight and small RNAs were carried out as previously described (22).

In vitro cleavage assay

MiRNA loaded AGO complexes were affinity purified from agroinfiltrated *N. benthamiana* leaves. Briefly, three days post-infiltration total leaf extracts were prepared as described above. The extracts were clarified by centrifugation and FLAG tagged AGO complexes were precipitated by M2 FLAG affinity gel. The bead bound complexes were washed with lysis buffer three times and subsequently with RISC buffer twice (40 mM HEPES [pH7.4], 100 mM potassium-acetate, 5 mM magnesium-acetate, 4 mM dithiothreitol). AGO complexes were eluted from the beads with 100 μg/ml FLAG peptide (Sigma-Aldrich). α-^{32}P-UTP labeled RNA substrates were prepared by *in vitro* transcription using T7 or SP6 RNA polymerases. The labeled RNA substrates were purified on 6% urea-PAGE. Approximately 1×10^4 CPM labeled substrate was used per cleavage reaction. The reaction mixtures were incubated at 30°C for 2 hours and subsequently resolved on a 6% or 8% urea-PAGE. The gels were dried onto filter paper and exposed to phosphor image screens. The screens were scanned with a Bio-Rad Pharos FX Plus Molecular Imager.

Bioinformatics analysis

Multiple alignments and phylogenetic tree constructions were done using the Vector NTI software package (In-

vitrogen). The I-TASSER webserver was used to predict three-dimensional protein structures (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (23). RNA secondary structures were predicted using the RNAfold webserver at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.

RESULTS

Cloning of *N. benthamiana* Argonautes

N. benthamiana is a widely used experimental host in plant biology, due mainly to the large number of viruses that can successfully infect it (24). Relying on the recent release of its draft genome sequence and various transcriptome data sets, we have cloned the predicted full-length cDNAs of the nine *N. benthamiana* AGO proteins (25–27). Phylogenetic comparison placed the *N. benthamiana* AGOs in the three major clades reported previously from other plant species (Supplementary Figure S1). The N domains of the proteins exhibit high degree of sequence divergence, most likely related to their different functions. On the other hand, the C terminal ends—especially the MID and PIWI domains—are much more conserved and an intact catalytic tetrad is also recognizable in all of the *N. benthamiana* AGOs (Supplementary Figure S2).

Establishment of an AGO2 specific transient reporter system

Despite its recently recognized crucial role in various stress responses, plant AGO2 is only poorly characterized. Thus we set out to study the AGO2 protein of *N. benthamiana* in greater details. First, we have established a transient *in vivo* reporter system to monitor the gene silencing abilities of AGO proteins. Our system is based on the *Agrobacterium*-mediated transient expression assay developed previously to analyze miRNA dependent gene silencing (28–30) (Supplementary Figure S3). The three components of the system are the following: sensor transcript, miRNA expression cassette and AGO expression cassette. These elements are incorporated into binary plasmid vectors to allow their efficient co-delivery into tobacco leaves by agroinfiltration. Our sensor plasmid encodes a GFP-Renilla luciferase fusion protein under the control of a constitutively active CaMV 35S promoter. MiRNA target sites are inserted into various positions of the sensor mRNA (3′ UTR or ORF). The second component of the reporter system encodes a miRNA. This plasmid also constitutively produces GFP protein, which is used as an internal control to normalize for the varying efficiencies of agroinfiltration. The AGO protein under study is expressed from the third binary plasmid. The RISC complexes assembled from the co-expressed AGO proteins and miRNAs can interact with the sensor mRNAs via the miRNA binding sites. Monitoring the expression of the sensor mRNA allows the assessment of gene silencing.

The ability of plant AGO1 to repress gene expression has been well established (31–33). Therefore, to test our system, first we have set up an AGO1 specific reporter assay (Figure 1A). We have inserted two binding sites of the known AGO1-associated miRNA, miR168 into the 3′ UTR of the sensor plasmid (34) (wild type sensor). A mutant sensor
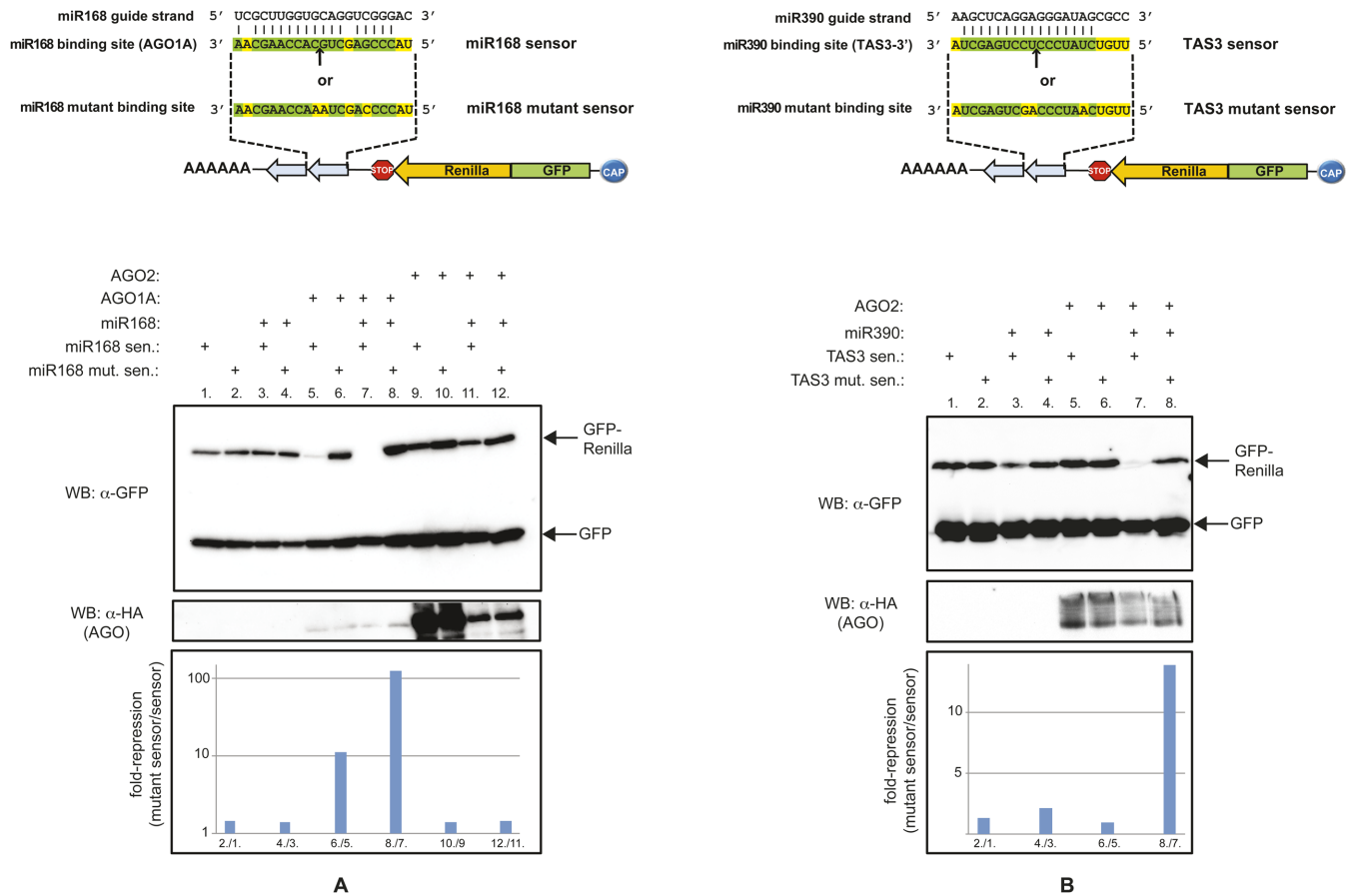
Figure 1. Establishment of AGO-dependent transient gene silencing assays. (**A**) To obtain an AGO1-specific sensor two copies of a miR168 binding site from the AGO1A gene were inserted into the 3′ UTR of the reporter construct. (**B**) AGO2-specific sensor was created by inserting two copies of a miR390 binding site from the TAS3 non-coding transcript into the 3′ UTR of the reporter construct. The predicted cleavage sites are indicated by arrows. Corresponding mutant sensors for each wild-type sensor were created by introducing mismatches adjacent to the cleavage site and in the 3′ supplementary region. Perfectly matched nucleotides are boxed in green, mismatches are boxed in yellow. *N. benthamiana* leaves were infiltrated with *Agrobacteria* strains as indicated. In some of the infiltrations the miRNA expressing *Agrobacteria* was replaced by equivalent amounts of *Agrobacteria* carrying the empty expression vector (pK7WG2D). Protein contents of the infiltrated leaves were analyzed by quantitative western blot using GFP antibody. The intensities of the 66 kDa GFP-Renilla fusion protein bands expressed by the sensors were normalized for the 30 kDa GFP bands expressed by the miRNA expression vector or the empty pK7WG2D plasmid. Target repression was calculated by dividing the normalized GFP-Renilla fusion protein band intensities obtained by the mutant sensor with that of the wild-type sensor. Expressions of the AGO proteins were also verified by probing the blots with HA antibody. Each reporter assays were repeated at least three times and a representative experiment is shown.

construct was also generated by introducing point mutations into the miR168 binding sites at positions 10, 11 and 16, which were predicted to abolish AGO1 mediated gene silencing (1–3). Using these sensors, gene silencing efficacy can be calculated by dividing the amount of GFP-Renilla luciferase fusion protein expressed by the mutant sensor with that of expressed by the wild-type sensor. Infiltration of the wild type or mutant sensor carrying *Agrobacteria* into tobacco leaves alone resulted in similar GFP-Renilla luciferase fusion protein levels (gene silencing efficacy ≈1). Co-expression of miR168 did not cause any change; however, in the presence of ectopic AGO1 the amount of the GFP-Renilla luciferase fusion protein produced by the wild type sensor dropped sharply compared to the mutant sensor (gene silencing efficacy >10). Co-delivery of AGO1 with miR168 synergistically increased gene silencing efficacy even further (>100). These findings have two implications: (i) the level of endogenous miR168 loaded AGO1 complexes is not sufficient to elicit significant degree of si-

lencing of the over-expressed reporters; (ii) no significant pool of free, programmable AGO1 exists in the cell. AGO2 co-expression with miR168 did not lead to gene silencing consistent with the observed binding preference of AGO2 for miRNAs starting with adenine (7) (miR168 starts with a uridine).

The above results confirmed that our system was suitable to monitor AGO mediated gene silencing *in vivo*. Next, we have set out to establish an AGO2 specific reporter system based on miR390 (Figure 1B). Although, miR390 was originally assumed to interact with AGO7 exclusively, newer data also show its significant association with AGO2 (35,36). To obtain a miR390 responsive reporter, we have introduced native miR390 binding sites—found at the 3′ end of the TAS3 non-coding transcript—into the 3′ UTR of our sensor. A mutant sensor was also generated by introducing mutations into the miR390 binding site, adjacent to the predicted cleavage site and in the 3′ supplementary region. The AGO2 sensor behaved similarly to the AGO1 sensor, in that

significant degree of gene silencing required co-expression of both the AGO protein and the corresponding miRNA (AGO2 and miR390). In addition, it was only responsive to AGO2 as its activity could not be significantly repressed by any of the *N. benthamiana* AGOs tested (AGO1A, AGO1B, AGO4A and AGO10) (Supplementary Figure S4). Thus, our system is suitable to monitor AGO2 activity with high specificity.
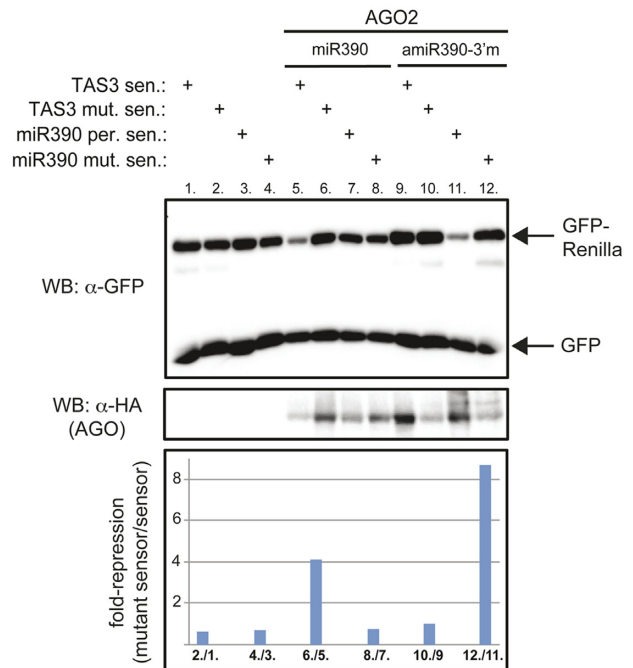
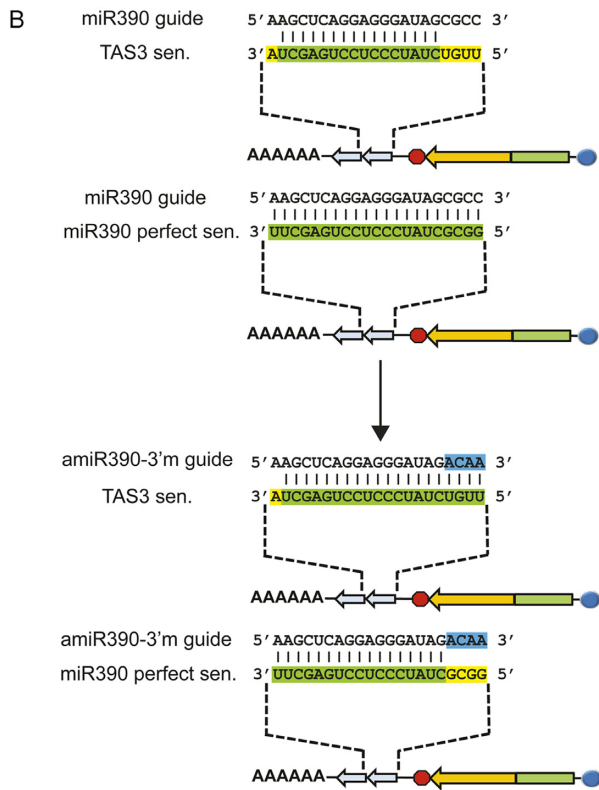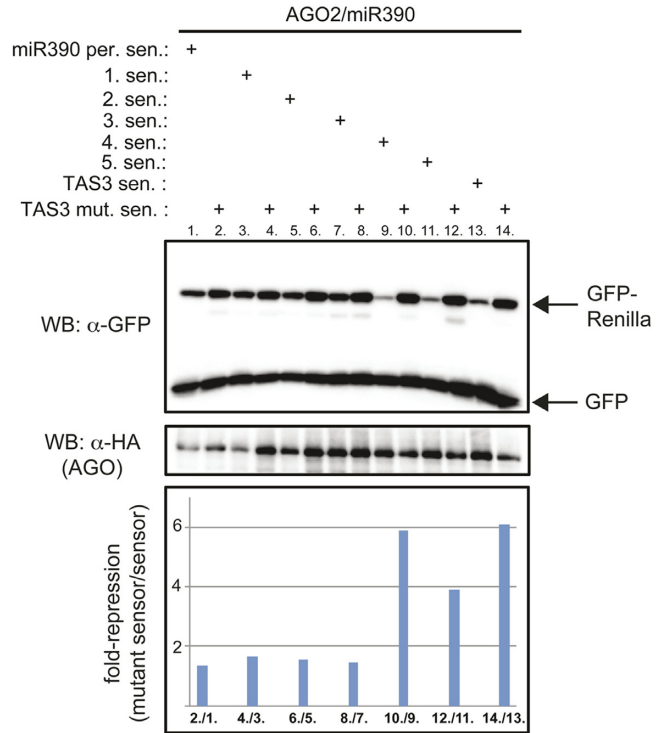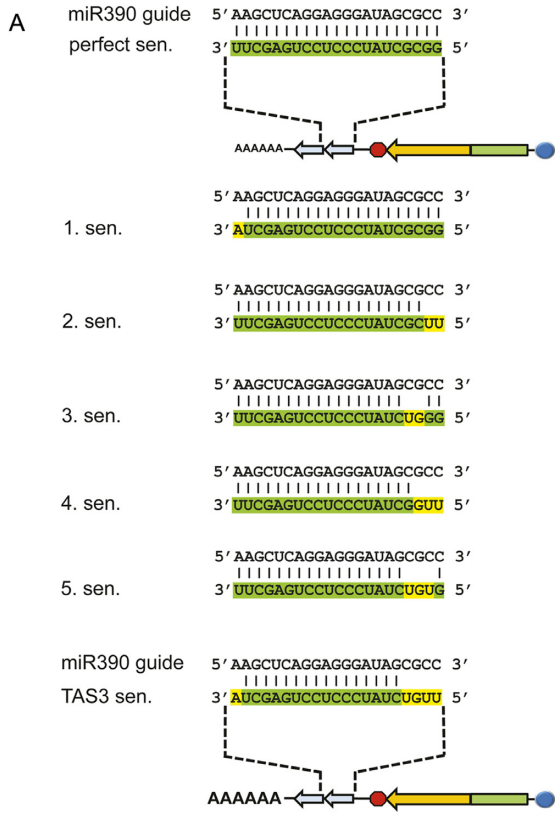### Pairing requirements for AGO2-mediated gene silencing

Earlier computational predictions indicated that plant miR-NAs show high degree of sequence complementarity with their target sites, leading to the general belief that extensive pairing is a necessary prerequisite for efficient gene silencing in plants (37–39). However, the 3′ miR390 binding site of the TAS3 transcript contains several mismatches relative to the miR390 guide strand. Since more than two consecutive mismatches are assumed to be detrimental to gene silencing, we tested whether correcting these mismatches can improve AGO2-mediated target repression (40). We inserted two miR390 binding sites, exhibiting 100% complementarity to the guide strand of miR390, into the 3′ UTR of the sensor plasmid. A corresponding mutant sensor was also created. Separate expression of miR390 or AGO2 did not elicit gene silencing on the perfectly complementary sensor and surprisingly, their co-expression was also ineffective (Supplementary Figure S5). Therefore, we systematically modified the TAS3 derived miR390 target site to unravel the sequential criteria for efficient AGO2-mediated silencing (Figure 2A). Restoring pairing between the 5′ terminal nucleotide of the miRNA guide strand and the 3′ end of the target site did not affect silencing efficacy. This finding is consistent with structural data indicating that the base of this nucleotide does not make contact with the target RNA (41). Further gradual modifications of the miR390 target site revealed that the presence of at least three mismatches at its 5′ end is necessary for efficient silencing. To confirm that these mismatches *per se* play a key role in target repression, we performed a complementary experiment (Figure 2B). We modified the 3′ end of the guide strand of miR390 to make it complementary with the TAS3 derived miR390 target site. Compensatory mutations were also introduced into the miR390 precursor to keep the original folding structure (Supplementary Figure S6). The modified miR390 (amiR390–3′ m) exhibited opposite gene silencing ability compared to the wild-type miRNA: it was ineffective on the natural TAS3 derived miR390 target site, while it was capable of efficiently silence the reporter containing binding sites perfectly complementary with the wild-type miR390 guide strand. These results clearly confirm that the presence of mismatches between the 3′ end of the miR390 guide strand and the 5′ end of the target site is a main determinant for efficient AGO2-mediated gene silencing.

Differences in the RISC/target mRNA stoichiometry may significantly affect the outcome of silencing. Therefore, we examined the silencing potential of perfectly complementary versus TAS3 derived miR390 binding sites, using different AGO/sensor ratios (Supplementary Figure S7). Importantly, even at the highest AGO/sensor ratio tested, dramatically weaker repression was observed on the perfectly complementary sensor than on the mismatch bearing sensor (≈3-fold repression versus >100-fold repression). These indicate that the perfectly complementary binding sites are inherently less efficient in mediating target repression than the mismatch bearing sites and even at high AGO/sensor ratios the former can not reach the silencing efficacy of the latter.

To extend our findings, we tested the pairing requirement for efficient target repression by another AGO protein and miRNA. First, miR390 was redirected into AGO1 by replacing the 5′ terminal adenine residue of the guide strand with a uridine. To preserve the original hairpin structure a compensatory nucleotide change was introduced into the appropriate position of the miR390 precursor (Supplementary Figure S6). Incorporation of the modified miR390 (miR390 A-U) into AGO1 was confirmed by IP-northern blot. Functionality of the miR390 A-U loaded AGO1 complexes was also verified in cleavage assays (Supplementary Figure S8). The miR390 programmed AGO1, like AGO2, was only able to silence the TAS3 derived mismatch bearing target, while it was ineffective on the perfectly complementary miR390 sensor (Figure 2C). Next, we tested target repression by a different AGO2 associated miRNA. The copper responsive miR408 starts with an adenine and is one of the most highly enriched miRNAs in AGO2 pull-downs (42,43). In addition, miR408 was also shown to associate with AGO1 (44). The miR408 target gene, Plantacyanin is regulated via a binding site, which has two mismatches at its 5′ end relative to the miR408 guide strand. To obtain a miR408 responsive reporter, two copies of this binding site were inserted into the 3′ UTR of the sensor plasmid (Supplementary Figure S9). A sensor perfectly complementary with the miR408 guide strand was also created. Corresponding mutant sensors (with mismatches adjacent to the cleavage site and in the 3′ supplementary region) served as references for assessing target repression. The Plantacyanin derived sensor was reproducibly repressed by both AGO1 and AGO2. Contrary, the perfectly complementary sensor was not responsive to any of the AGOs. Therefore, mismatches between the 3′ end of the miRNA guide strand and the 5′ end of the target site increased silencing efficacy by both AGO1 and AGO2 regardless of the miRNA used for their programming.

Next, we sought to understand the differential activities of AGO2 toward mismatch bearing and perfectly complementary targets. Several recent reports have indicated reciprocal relationships between small RNAs and their regulated targets (45–47). For instance, highly complementary target RNAs were shown to decrease silencing efficacy by accelerating the release of guide strands from RISCs and/or by triggering their non-templated 3′-end tailing and subsequent decay. To test whether similar factors influence gene silencing in our system, we have co-expressed miR390 and AGO2 with increasing quantities of TAS3 (mismatch bearing) or perfectly complementary miR390 target site containing sensors. None of the sensors affected total miR390 levels and importantly the binding of the miR390 guide strand with AGO2 was not changed either (Supplementary Figure S10). Thus, a highly complementary target does not seem to affect the stability of miR390 and its association with AGO2.
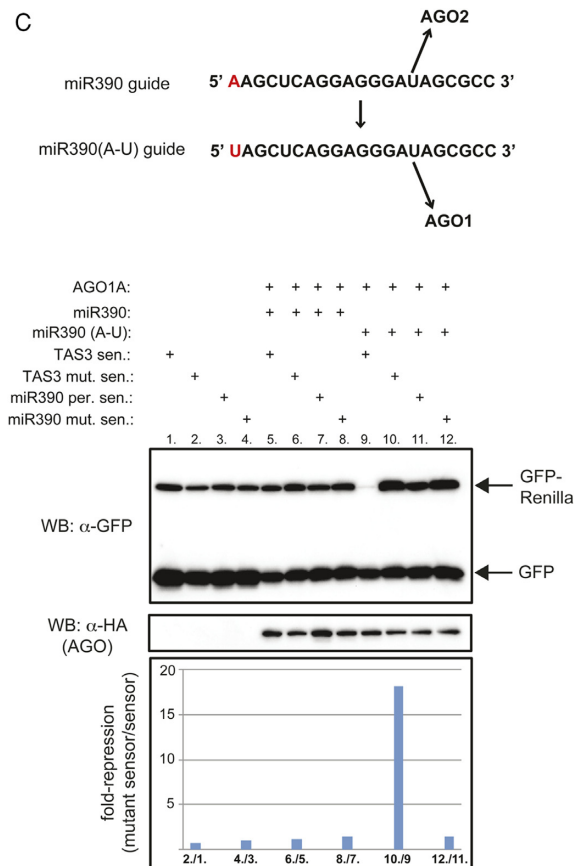
Figure 2. Pairing requirements for AGO2-mediated target repression. (**A**) The miR390 binding site was modified in a step-wise fashion to determine the minimal sequence requirement of efficient AGO2-mediated target repression. On the left schematic structures of the sensors are shown. (**B**) Modification of the 3′ end of miR390 guide strand can reverse its gene silencing ability. On the left schematic structures of the sensors and miRNAs are shown. (**C**) Redirection of miR390 into AGO1 shows that AGO1 has similar target preferences as AGO2. Reporter assays were repeated at least three times and a representative experiment is shown.

Previous data suggested that miRNA-mediated regulation is chiefly accomplished by AGO-catalyzed slicing of the target RNA (48,49). Consequently, the differential silencing of mismatch bearing and perfectly complementary targets may reflect their differential cleavage by AGOs. To examine this possibility *in vitro* target slicing assay was established. MiR390 programmed affinity purified AGO2 efficiently cleaved a 270-nt segment of the TAS3 transcript containing a miR390 binding site. When the mismatches, which were shown to inhibit gene silencing *in vivo* were introduced into the TAS3 substrate (the mismatches found in the mutant sensor), target slicing was completely abolished (Figure 3). Interestingly, conversion of the native miR390 binding site to a perfectly complementary site also severely compromised target cleavage. These results suggest that the susceptibility of a miR390-binding site to be silenced *in vivo* shows a positive correlation with its sensitivity toward AGO2-mediated cleavage *in vitro*.

Mechanisms of AGO2-mediated gene silencing

MiR390 was originally described as an AGO7 associated miRNA. It was shown to play an essential role in tasiRNA biogenesis by helping the recruitment of RDR6 to the TAS3 non-coding transcript (50,51). RDR6 is also required for transgene derived secondary siRNA production thus, we examined the possibility whether this pathway is also involved in gene silencing in our reporter system (52). Co-expression of AGO2 and miR390 with the sensors in RDR6 knock-down *N. benthamiana* (53) gave essentially the same result as in wild-type plants (Supplementary Figure S11). Hence, the over-expressed miRNAs act directly on the sensor transcripts and RDR6-dependent secondary siRNAs do not contribute to target repression in our transient assays.

In plants most experimentally verified miRNA binding sites are located in ORFs (54,55). AGO1-RISC complexes have been shown to work more efficiently on ORF sites than on 3′ UTR sites both *in vitro* and *in vivo* (19,30,56). Therefore, we tested whether moving the miR390 binding sites into the ORF (into the linker region connecting the GFP and Renilla luciferase domains of the sensor), may also increase AGO2-dependent target repression (Figure 4). Indeed, we found that TAS3 derived miR390 sites bestowed significant degree of silencing upon the ORF sensor even in the absence of ectopic AGO2 or miR390. Co-expression of the miRNA and the AGO protein led to an even more pronounced effect (Figure 4A lanes 1–4 versus Figure 4B lanes 1–4). Unlike in their 3′ UTR location, perfectly complementary miR390 sites were also able to elicit target re-
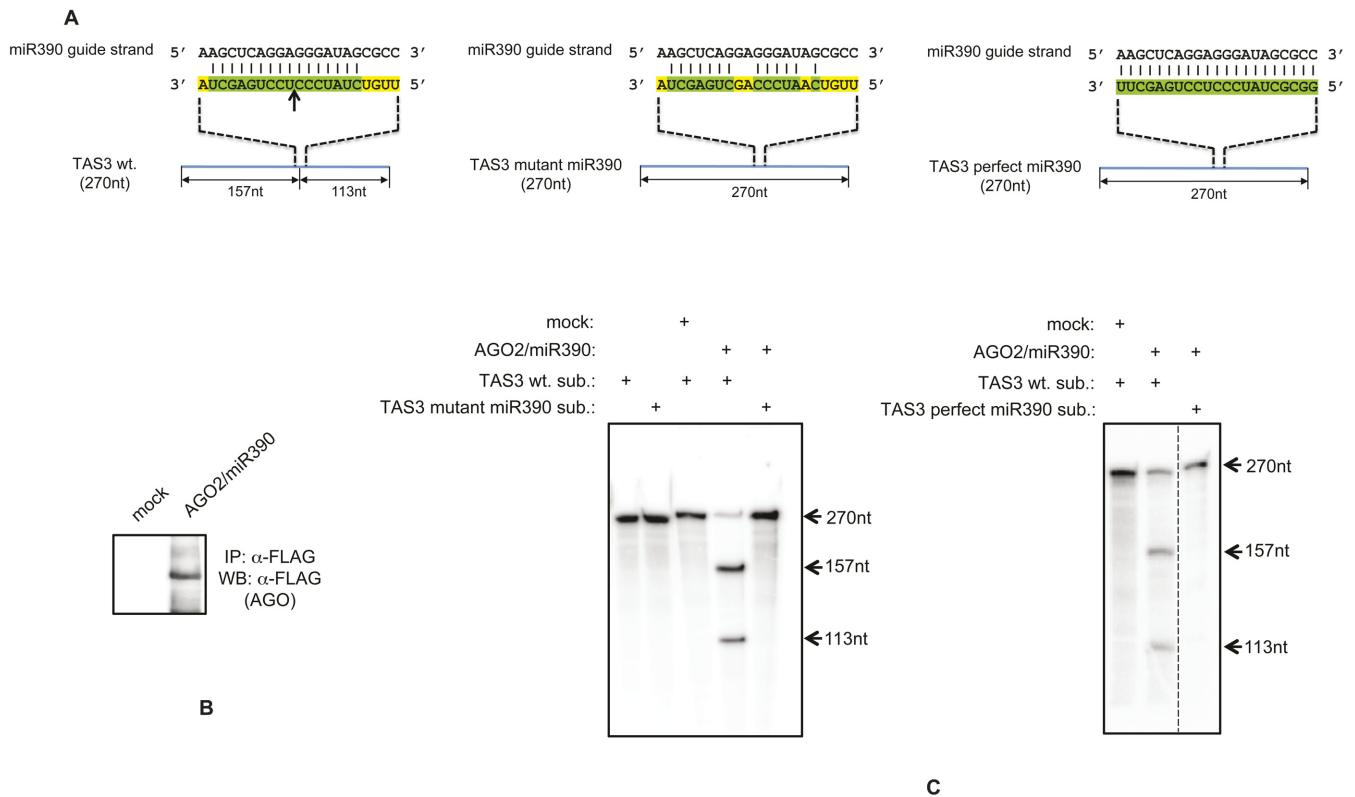
Figure 3. Analysis of cleavage activity of AGO2 by *in vitro* cleavage assays. (**A**) Structures of the RNA substrates used in the *in vitro* cleavage assays. The expected cleavage site is indicated by an arrow. (**B**) MiR390 programmed AGO2 complexes used for the cleavage assays were produced in agroinfiltrated *N. benthamiana* leaves. The complexes were immunopurified by FLAG affinity resin and analyzed by western blot. (**C**) Analysis of the cleavage products by denaturing acrylamide gel electrophoresis. The sizes of the expected products are indicated.

pression in the ORF. However, they were reproducibly less effective than the mismatch bearing TAS3 derived sites, as significant degree of silencing in this constellation required the presence of exogenous AGO2/miR390 (Figure 4A lanes 5–8 versus Figure 4B lanes 5–8). In summary, similarly to AGO1, AGO2 containing silencing complexes work more efficiently on ORF binding sites (even on perfectly complementary miR390 binding sites) than on 3′ UTR sites.

Plant miRNAs recognize highly complementary target sites and accordingly, earlier models assumed AGO-mediated slicing as their main mode of action. Recent results, however, have indicated that translational repression with limited mRNA decay or cleavage may be more prevalent in plants than previously believed (19,56). Therefore, we tested the contribution of translational repression versus mRNA degradation to gene silencing in our reporter system (Figure 4). We found no strict correlation between the protein levels and the mRNA levels. Frequently, even a robust drop in the protein level (more than 1 order of magnitude) was not accompanied by a discernible change at the transcript level. Subsequently, we also examined whether the slicer defective AGO2(D907A) can silence gene expression (Supplementary Figure S12). The mutant protein was unable to elicit gene silencing on miR390 binding sites inserted either in the 3′ UTR or the ORF. Interestingly, however, it almost completely abolished target repression mediated by endogenous AGO complexes in a dominant negative fash-

ion. Therefore, even though a significant degree of AGO2-mediated mRNA degradation cannot be detected, the catalytic activity of AGO2 is still a necessary prerequisite for efficient gene silencing.

Identification of functionally important AGO2 residues

Next, we wanted to further characterize AGO2 by identifying functionally important regions of the protein. Since the core biochemical properties of AGOs are evolutionarily conserved, we used multiple sequence alignments between evolutionarily distant AGO proteins to pinpoint regions of potential functional significance. We identified amino acid residues of AGO2, which were predicted to affect three main aspects of AGO function: catalytic activity, protein–protein interactions and small RNA binding (Figure 5A). First, we identified the four acidic amino acids of AGO2 that constitute the DEDD catalytic tetrad. Earlier data have shown that changing any of these residues to an A results in a catalytically inactive AGO protein (7–9). In agreement, the AGO2(D907A) mutant was not able to silence the sensors in reporter assays (Figure 5B). As expected this mutant was also inactive in the *in vitro* cleavage assay although it could efficiently bind miR390 (Figure 5C, D).

A number of proteins have been identified, which can modulate various functions of RISCs. Many of them employ GW-rich domains for directly binding to AGOs (57–64). Structural studies have shown that these so-called
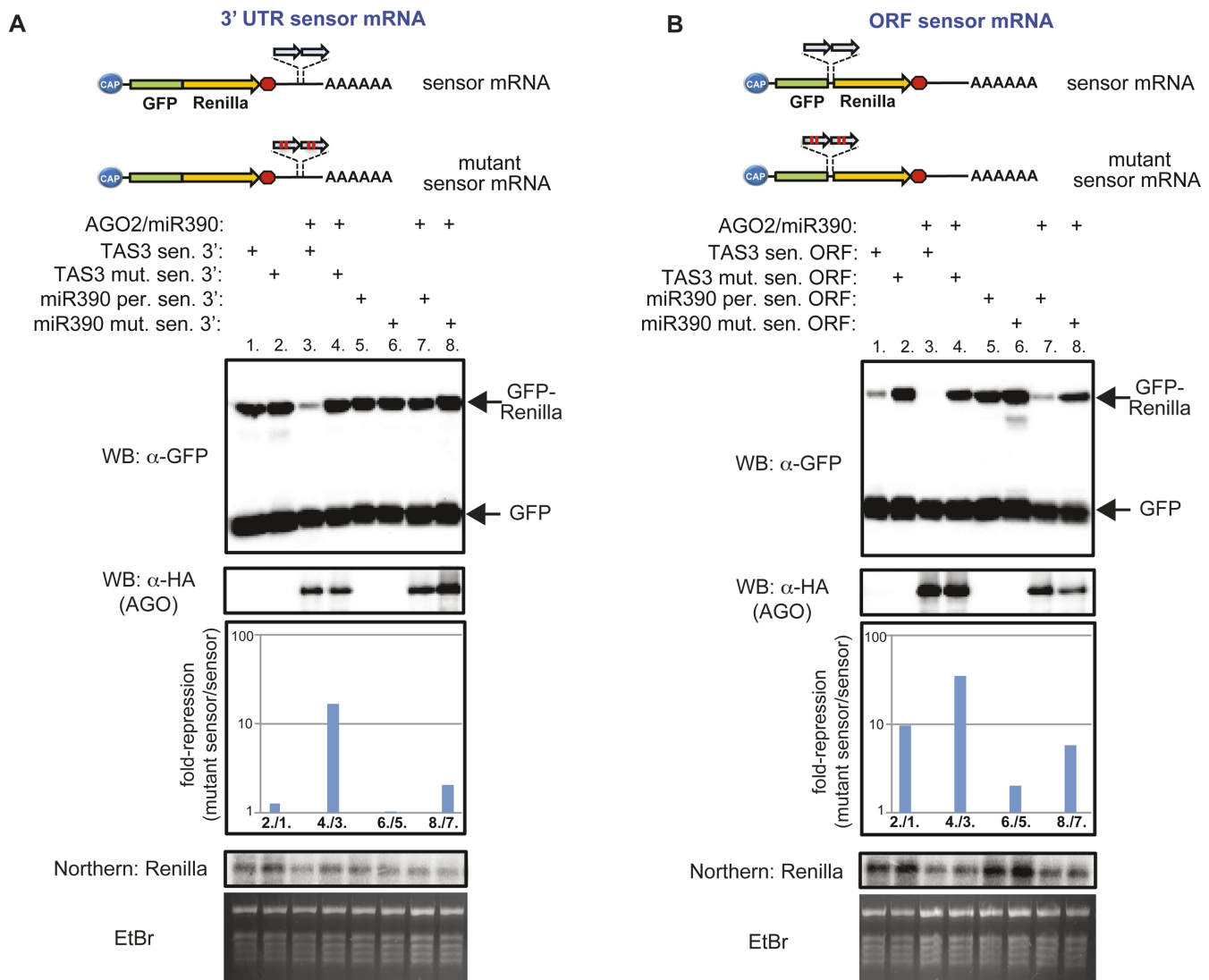
Figure 4. Effect of the target site position on AGO2-mediated gene silencing. The cartoons on the top show the schematic structures of the 3′ UTR (**A**) or ORF (**B**) sensors used. Western blot analysis of the protein lysates prepared from the infiltrated *N. benthamiana* leaves (first and second panels). The charts in the third panel show the normalized quantitation of the GFP western blots (translational repression). Total RNA samples were prepared from the same infiltrated leaves and the expression of the GFP-Renilla mRNA was analyzed by northern blot (fourth panel). On the fifth panel EtBr stained agarose gel is shown as loading control. Reporter assays were repeated at least three times and a representative experiment is shown.

AGO-hooks fit into a specific binding pocket of AGO proteins (9,65,66). It is hypothesized that in animals this binding site is freely available whereas in plants, accessibility to this pocket is restricted by an adjacent W residue (Supplementary Figure S13A) (9). To assess the role of GW-repeat proteins in the regulation of AGO2 we have generated two mutant molecules. In one of them we have altered the putative GW-binding pocket by replacing P899 with an S residue. P899 of *N. benthamiana* AGO2 is equivalent with P840 of *A. thaliana* AGO1 (Supplementary Figure S13B). The P840S change in *A. thaliana* AGO1 results in the functionally compromised *ago1–26* allele (67). In the second mutant we replaced the W857 residue with an A, which according to structural modeling regulates the accessibility of the GW-binding pocket in plant AGOs (9). In reporter assays both proteins were able to silence gene expression; however,

the W857A mutant was reproducibly less active than the wild type AGO2 (Figure 5B). MiR390 binding and *in vitro* cleavage activities of the mutant proteins closely paralleled the tendencies of the gene silencing assays (Figure 5C, D). In summary, our results suggest that AGO2 interacts with various AGO-hook containing proteins, which can modulate its activity in a complex combinatorial manner.

The N terminal domain of AGOs exhibit significant degree of sequence divergence nevertheless, recent data on human AGO2 underscored the importance of this region in such key processes as small RNA loading and RISC maturation (68). Two amino acid residues of human AGO2, D95 and F181 were shown to play crucial roles in the above processes. The equivalent amino acid positions of *N. benthamiana* AGO2 were identified (D328 and F412) and mutated to As along with adjacent residues, which also show high
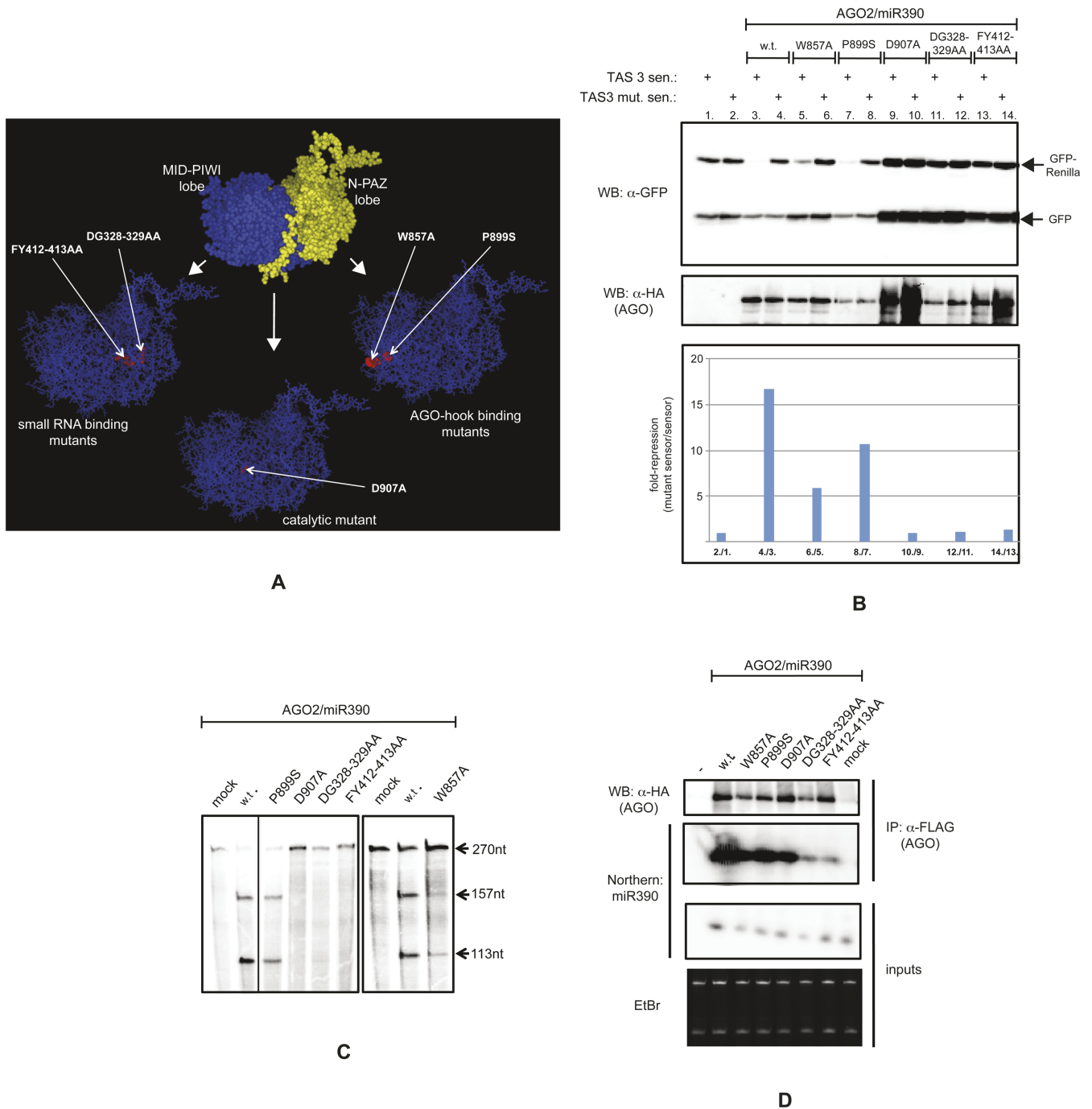
Figure 5. Functional analysis of AGO2 mutants. (**A**) I-TASSER modeling of *N. benthamiana* AGO2 protein. Similarly to other AGOs the *N. benthamiana* AGO2 protein exhibit the characteristic bilobal structure. The MID-PIWI lobe (blue) and the N-PAZ lobe are indicated (yellow). Based on similarities between AGO proteins from evolutionarily distant organisms, functionally important amino acid residues of AGO2 were identified. These amino acids were predicted to affect three main features of AGO function: catalytic activity, protein–protein interactions and small RNA binding. Mutants were generated in the identified amino acid positions (labeled with red). (**B**) Analysis of the gene silencing ability of the various AGO2 mutants using the transient agroinfiltration assay. Reporter assays were repeated at least three times and a representative experiment is shown. (**C**) Cleavage activity of AGO2 mutants was tested *in vitro* using radioactively labeled 270-nt segment of the TAS3 non-coding transcript. The sizes of the expected cleavage products are indicated. (**D**) Small RNA binding of AGO2 mutants was assessed by their co-precipitation with miR390. The mutant proteins were co-expressed with miR390 in tobacco leaves. Subsequently, AGOs were affinity purified on FLAG beads and the co-precipitating miR390 was analyzed by northern blot. The top panel shows the precipitated AGO2 proteins (western blot). On the second panel AGO2 associated miR390 was detected by northern blot. The bottom two panels are input controls (third panel northern blot, fourth panel EtBr stained gel).
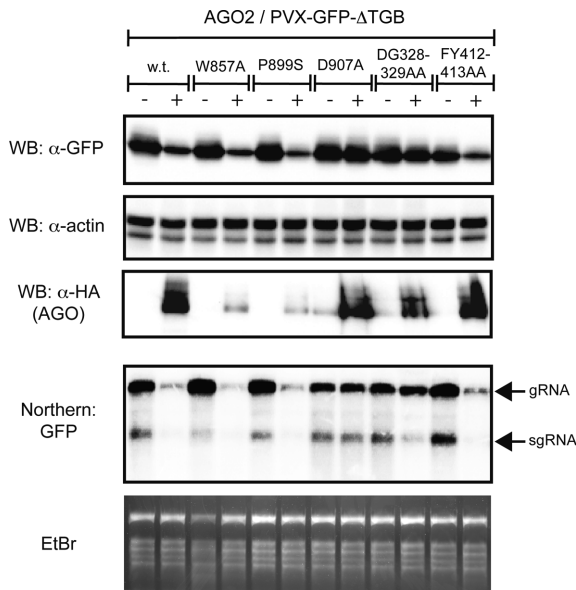
Figure 6. Antiviral activity of AGO2. *N. benthamiana* leaves were infiltrated with PVX-GFP-ΔTGB together with pBIN61 'empty' vector (left side) or the indicated AGO2 expression construct carrying Agrobacteria (right side). On the top panel expression of the virally encoded GFP was analyzed by western blot. As a loading control the same blot was probed with an actin antibody (second panel). Expression of AGO2 was analyzed by HA antibody (third panel). Abundance of viral RNA was analyzed by northern blot (fourth panel). On the fifth panel EtBr stained agarose gel is shown as loading control.

degree of conservation in various AGOs (Supplementary Figure S13C). The mutant proteins (DG328–329AA and FY412–413AA) were tested for their ability to silence gene expression, load miRNAs and cleave targets (Figure 5B–D). Both proteins were strongly defective in the above assays, indicating that the structural requirements for miRNA loading and RISC maturation are similar between plants and animals.

Antiviral activity of AGO2

Finally, we wanted to examine how the identified mutations affect the antiviral activity of AGO2. Recently, it was reported that transient over-expression of *A. thaliana* AGO2 in tobacco leaves limited the replication of potato virus X (PVX) (15). To test whether *N. benthamiana* AGO2 possess similar activity, we have co-delivered the VSR deficient form of PVX (PVX-GFP-ΔTGB) and AGO2 by agroinfiltration into tobacco leaves and subsequently monitored virus accumulation (Figure 6). The replication of the virus was strongly compromised in the presence of AGO2 as evidenced by the reduced levels of virally encoded GFP and viral genomic RNA. Next, we tested the ability of mutant AGO2 molecules to inhibit virus accumulation. While the W857A and P899S mutants exhibited strong antiviral activity, the catalytically inactive D907A mutant was completely inactive in this respect. Unexpectedly, of the N domain mutants the FY412–413AA mutant kept its ability to restrict PVX replication. This suggests that unlike miRNA loading, vsiRNA loading is not compromised in this AGO2 mutant. In summary, *N. benthamiana* AGO2 possess antiviral activ-

ity similarly to its *A. thaliana* ortholog, which depends on its catalytic activity and small RNA binding.

DISCUSSION

Until recently the role of plant AGO2 has been obscure. It was assumed that its main function is to sequester small RNAs away from other AGOs and thereby indirectly regulate their activities. Newer studies however have uncovered more active roles for AGO2 in RNA silencing. By collaborating with AGO1, AGO2 silences non-conserved intergenic regions, pseudogenes and transposons (62,63). In addition, it has also been shown to function independently in various anti-pathogenic defense pathways and genotoxic stress responses (11–18). Considering the significance of the above processes it is important to functionally characterize a plant AGO2 protein in greater details. We chose to study the AGO2 protein of *N. benthamiana* because, this plant is amenable to a number of powerful molecular biological and biochemical techniques and it is also susceptible to a wide range of plant pathogens. Many of the recently described activities of AGO2 may invoke its ability to repress gene expression. Thus, we have developed an agroinfiltration-based transient expression assay to directly assess the gene silencing activity of AGO2. Our reporter system is based on miR390 binding site containing sensor transcripts. MiR390 was previously believed to be an exclusively AGO7-associated miRNA; however, recent data have also revealed its significant enrichment in AGO2 IP fractions (35,36). Indeed our miR390 sensors responded robustly and specifically to the co-expression of AGO2 and miR390, but none of the other tested AGOs (AGO1A, AGO1B, AGO 4A, AGO10) affected their activities significantly. Likely, the reporters would have also been responsive to AGO7; however, we could not test this possibility due to difficulties in the *in vivo* over-expression of the *N. benthamiana* AGO7 protein. Nonetheless, since the endogenous AGO7 activity is undetectable in *N. benthamiana* leaves the employed sensors are suitable to follow AGO2 activity *in vivo* with high specificity and sensitivity.

We observed that AGO2 was able to repress gene expression via miRNA binding sites located either in the 3′ UTR or ORF, with a considerably stronger effect on the latter. This observation is similar to those made with AGO1 and it may be related to the divergent repression mechanisms acting on the topologically different target sites as was suggested earlier—inhibition of translation initiation on 3′ UTR sites versus steric hindrance on ORF sites (19). With the more widespread use of antibodies specifically recognizing plant proteins, it is becoming more obvious that slicing independent translational repression is also common in plants (19,56). Indeed, we also found that during AGO2 mediated gene silencing the protein and mRNA levels were not moving in parallel. Frequently, even a robust drop at the protein level was not accompanied by a comparable change at the mRNA level. Although, in our system we follow the expression level of a reporter protein a similar observation was made previously by monitoring the expression of the natural AGO2 target gene MEMB12 (17). This suggests that slicing independent translation inhibition is indeed being used by AGO2. Despite the lack of a significant degree

of mRNA degradation, we found that the catalytic activity of AGO2 was still indispensable for efficient gene silencing. To reconcile these apparently contradictory results, one may assume that a step other than target slicing is dependent upon the catalytic activity of AGO2. Indeed, according to a recent finding AGO7 mediated miR390* strand cleavage is essential for the maturation of the AGO7-miR390 RISC (69). Likely, the maturation of AGO2-miR390 complexes happens in a similar manner, thereby explaining the need for the catalytic activity of AGO2 for target repression.

Another issue that needs further consideration is the apparent necessity for base pairing around position 10 and 11 between the miRNA guide strand and the target RNA for efficient silencing by AGO2. How one can explain the need for base pairing in this region if no target cleavage is necessary for translational repression? A possible explanation comes from structural studies indicating that efficient cleavage of a target requires that the guide base pairs at least five to eight nucleotides beyond the seed (nt. 2–8) (70,71). This so-called 'zippering' of the guide-target helix induces a conformational change in the AGO protein thereby it can attain a catalytically competent state. Possibly, a similar conformational change may help the recruitment of various factors by AGOs necessary for cleavage-independent translational repression as well. If so, mismatches between nt. 9–16 may inhibit not only cleavage-dependent but also cleavage-independent target repression.

Interestingly, base pairing in another segment of the guide-target duplex affects the gene silencing ability of AGO2 differently. The natural miR390 binding site (derived from the TAS3 non-coding RNA) carries mismatches at its 5′ end relative to the miR390 guide strand. Surprisingly, correcting these mismatches severely compromised AGO2-mediated gene silencing instead of improving it. Interestingly, a miR390 binding site, which was perfectly complementary with the miR390 guide strand, was also sliced much less efficiently than the mismatch bearing native site. These observations suggest that AGO2 function is reduced on perfectly complementary target sites regardless of whether it involves target cleavage or translational repression. Importantly, this feature is not limited to the AGO2/miR390 complex as AGO2 loaded with miR408 and AGO1 programmed either with miR390 or miR408 exhibited similar target preferences. The diminished AGO function was not a consequence of the perfectly complementary target induced accelerated unloading and/or degradation of guide strands described earlier in animal RISC complexes (43–47). Instead, a more likely explanation is that extensive miRNA/target pairing, which spreads over to the 3′ tail region (nt 18–21), slows down the dissociation of AGO complexes from the binding sites, thereby decreasing the turnover rate of catalysis. This hypothesis is in agreement with recent single molecule studies on RISC-target interactions, which show that the attachment of the 3′ end of the guide strand to the PAZ domain of AGO reduces the binding affinity between miRNA and target RNA (72). The diminished RISC-target interaction facilitates the release and possibly recycling of AGOs. Limited mobility of the tail region of the guide strand on a perfectly complementary target site may hinder its association with the PAZ domain thereby, resulting in slower target release and as

a consequence decreased RISC activity. In addition to the slow recycling rate of RISCs, other factors may also participate in the poor silencing outcome on perfectly complementary sensors. Even at high RISC/target ratios, perfectly complementary sensors are repressed much less efficiently than mismatch bearing sensors. This observation indicates that inefficient recruitment of silencing components by perfectly complementary biding sites may also contribute to their reduced activity. Nonetheless, our findings are consistent with the hypothesis that AGO proteins divide the small RNA guide into domains with distinct biochemical and biophysical properties, which affect gene silencing differently (73,74).

In plants, besides regulating gene expression, AGO proteins are also the main executors of antiviral defense. Accordingly, we found that *N. benthamiana* AGO2 was capable of curtailing the amplification of the VSR deficient form of PVX. AGO2-dependent gene silencing and viral restriction exhibited important similarities, although significant differences could also be noticed. While both processes were strictly dependent on the small RNA binding ability and catalytic activity of AGO2, only during the later could strong correlation be observed between the abundance of RNA (viral RNA) and its encoded protein product (virally encoded GFP). These observations indicate that *N. benthamiana* AGO2 possess the ability to inhibit its target RNAs via both slicing-dependent and -independent mechanisms. Understanding the molecular bases of these two *modi operandi* of AGO2 will be an important future task.

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