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Rapid paper

The Mechanism Selecting the Guide Strand from Small RNA Duplexes is Different Among *Argonaute* Proteins

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Double-stranded RNA induces RNA silencing and is cleaved into 21–24 nt small RNA duplexes by Dicer enzyme. A strand of Dicer-generated small RNA duplex (called the guide strand) is then selected by a thermodynamic mechanism to associate with Argonaute (AGO) protein. This AGO–small RNA complex functions to cleave mRNA, repress translation or modify chromatin structure in a sequence-specific manner. Although a model plant, *Arabidopsis thaliana*, contains 10 AGO genes, their roles and molecular mechanisms remain obscure. In this study, we analyzed the roles of *Arabidopsis AGO2* and *AGO5*. Interestingly, the 5′ nucleotide of small RNAs that associated with AGO2 was mainly adenine (85.7%) and that with AGO5 was mainly cytosine (83.5%). Small RNAs that were abundantly cloned from the AGO2 immunoprecipitation fraction (miR163-LL, which is derived from the Lower Left of mature miR163 in pre-miR163, and miR390) and from the AGO5 immunoprecipitation fraction (miR163-UL, which is derived from the Upper Left of mature miR163 in pre-miR163, and miR390*) are derived from the single small RNA duplexes, miR163-LL/miR163-UL and miR390/miR390*. Each strand of the miR163-LL/miR163-UL duplex is selectively sorted to associate with AGO2 or AGO5 in a 5′ nucleotide-dependent manner rather than in a thermodynamic stability-dependent manner. Furthermore, we showed that both AGO2 and AGO5 have the ability to bind cucumber mosaic virus-derived small RNAs. These results clearly indicate that the mechanism selecting the guide strand is different among AGO proteins and that multiple AGO genes are involved in anti-virus defense in plants.

Keywords: *Arabidopsis* — *Argonaute* (AGO) — *Cucumber mosaic virus* (CMV) — MicroRNA (miRNA) — 5′ Nucleotide of small RNA — RNA silencing.

Abbreviations: AGO, Argonaute; CMV, cucumber mosaic virus; HA, hemagglutinin; IP, immunoprecipitation; miRNA, microRNA; nat-siRNA, siRNA derived from a pair of natural *cis*-antisense transcripts; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; tasiRNA, *trans*-acting siRNA; vi-siRNA, virus-derived siRNA.

Introduction

RNA silencing is a small RNA-mediated gene regulatory mechanism conserved among eukaryotes. Double-stranded RNA induces RNA silencing and is cleaved into 21–24 nt small RNA duplexes by Dicer enzyme. Dicer-generated small RNA then associates with Argonaute (AGO) protein, and the RNA-induced silencing complex (RISC) containing the AGO–small RNA complex functions to cleave mRNA, repress translation or modify chromatin structure in a sequence-specific manner (Brodersen and Voinnet 2006, Tolia and Joshua-Tor 2007). The process for forming an AGO–small RNA complex consists of two steps: small RNA duplex selection between AGO proteins and subsequent 'guide strand' selection in each AGO protein.

Most eukaryotes, except for yeast, encode multiple AGO genes in their genomes; for example, fly (*Drosophila melanogaster*), human and plant (*Arabidopsis thaliana*) have two, four and 10 AGO genes, respectively (Tolia and Joshua-Tor 2007). In *D. melanogaster* and *Caenorhabditis elegans*, it has been shown that sorting of small RNA is dependent on the intrinsic structure (with or without mismatch in a central region) of the small RNA duplex (Steiner et al. 2007, Tomari et al. 2007).

The strand of the small RNA duplex that assembles with the AGO protein is called the 'guide strand' and the other strand is called the 'passenger strand'. The guide strand is selected from the small RNA duplex depending on the thermodynamic differences between the two ends of the small RNA duplex (Khvorova et al. 2003, Schwarz et al. 2003). In *D. melanogaster*, an RNA-binding protein R2D2,

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which recognizes the end of the small RNA duplex with a relatively higher thermodynamic stability (Tomari et al. 2004), is important for the selection of the guide strand in AGO2, whereas the mechanism whereby the AGO proteins of other eukaryotes recognize the guide strand remains obscure.

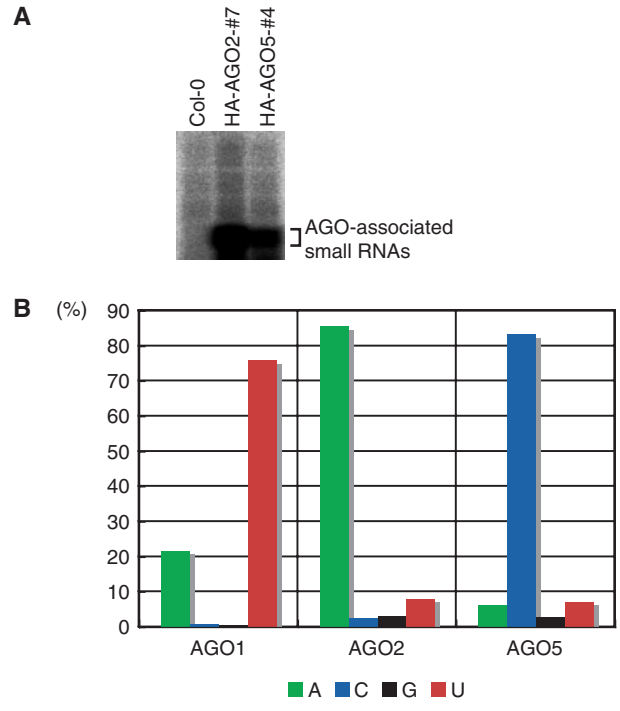
Of 10 AGO genes in *Arabidopsis*, the roles of *AGO1*, *AGO4*, *AGO6*, *AGO7* and *AGO10* have been investigated mainly by genetic analysis (Brodersen and Voinnet 2006, Vazquez 2006, Zheng et al. 2007). Recently, small RNAs associated with AGO1 and AGO4 have been characterized (Baumberger and Baulcombe 2005, Pontes et al. 2006, Qi et al. 2006). *AGO1* is mainly involved in the microRNA (miRNA) pathway and *AGO4* is mainly involved in DNA and chromatin modification, leading to transposon silencing (Vazquez 2006). The roles of the remaining *Arabidopsis* AGO genes remain obscure.

In this study, we analyzed the roles of *Arabidopsis* *AGO2* and *AGO5*. Interestingly, the 5′ nucleotide of small RNAs that associated with AGO2 was mainly adenine and that of AGO5 was mainly cytosine. Small RNAs that were abundantly cloned from the AGO2 fraction (miR163-LL, which is derived from the Lower Left of mature miR163 in pre-miR163, and miR390) and from the AGO5 fraction (miR163-UL, which is derived from the Upper Left of mature miR163 in pre-miR163, and miR390*) are derived from the single small RNA duplexes, miR163-LL/miR163-UL and miR390/miR390*. Furthermore, each strand of the miR163-LL/miR163-UL duplex is selectively sorted to associate with AGO2 or AGO5 in a 5′ nucleotide-dependent manner. These results indicate that the mechanism for selecting the guide strand is different among AGO proteins.

Results and Discussion

To examine roles of *Arabidopsis* AGO genes, we selected and established T-DNA insertion lines of all AGO genes except for *AGO1* and *AGO4* (Supplementary Figs S1, S2) and examined the accumulation of small RNA in inflorescent tissue of these mutants. We did not find an alteration of accumulation of small RNA (Supplementary Fig. S3) except for *TAS3* *trans*-acting small interfering RNA (tasiRNA) (ta-siR2142) in *ago7* and transposon-derived siRNAs (Simplehat2) in *ago6*, as those have been reported previously (Adenot et al. 2006, Fahlgren et al. 2006, Zheng et al. 2007).

To examine the roles of AGO genes, therefore, we generated a series of transgenic *Arabidopsis* plants expressing the respective AGO proteins with an N-terminal hemagglutinin (HA) epitope. Of these, we selected HA-AGO2 and HA-AGO5 plants for further analysis. First, to examine what small RNAs are associated with AGO2 and AGO5, we carried out immunoprecipitation (IP) from inflorescent tissue. Before cloning of small RNA, we confirmed successful IP of AGO2- and AGO5-associated small RNAs by 5′ end labeling of immunoprecipitated small RNAs (Fig. 1A). We cloned and then sequenced AGO2- and AGO5-associated small RNAs. We determined the sequences of 611 (AGO2) and 559 (AGO5) small RNA clones that varied in length from 18 to 26 nucleotides. Of these clones, 532 (87.1%, AGO2) and 484 (86.6%, AGO5) completely matched sequences in the *Arabidopsis* genome. Only small RNAs (1,016 clones) that completely matched the genomic sequence were analyzed further.



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	sRNA name	No. sequenced	%
AGO2	miR163-LL	39	7.3
	miR390	30	5.6
	miR172	22	4.1
AGO5	miR163-UL	16	3.3
	miR390*	7	1.4

Fig. 1 Cloning of *Arabidopsis* AGO2- and AGO5-associated small RNAs. (A) 5′-End-labeled AGO2- and AGO5-associated small RNAs were separated by urea-PAGE and then detected by autoradiography after 5′ end labeling with [^{32}P]ATP. Before the 5′ end labeling, small RNAs were treated with bacterial alkaline phosphatase. Lines HA-AGO2-#7 and HA-AGO5-#4 expressed HA-AGO2 and HA-AGO5 proteins, respectively. (B) Ratios of the first 5′ nucleotide in AGO1-, AGO2- and AGO5-associated small RNAs. The data of AGO1-associated small RNAs were derived from Qi et al. (2006). (C) The most numerous small RNAs cloned from the AGO2 fraction and from the AGO5 fraction.

Interestingly, AGO2- and AGO5-associated small RNAs showed a strong preference for adenine (85.7%) and cytosine (83.5%) nucleotides, respectively, at their first 5′ end (Fig. 1B and Supplementary Tables S1, S2). In the AGO2 fraction, the ratio of adenine at the first 5′ end was significantly higher than that in whole AGO2-associated small RNA sequences ($P = 6.1 \times$ E-151, χ^2 test). In the AGO5 fraction, the ratio of cytosine at the first 5′ end was significantly higher than that in whole AGO5-associated small RNA sequences ($P = 3.4 \times$ E-170, χ^2 test). In addition, we checked 5′ nucleotides of AGO1-associated small RNAs, which were previously reported (Qi et al. 2006), and found that AGO1 has a preference for the nucleotide uridine (76.1%) at the first 5′ end (Fig. 1B). Collectively, these results suggest that *Arabidopsis* AGO1, AGO2 and AGO5 have a strong preference for uridine, adenine and cytosine nucleotides, respectively, at the first 5′ end of their associated small RNAs. As far as we know, AGO2 and AGO5 are the first examples of AGO proteins having a 5′ preference other than for uracil in the associated small RNAs.

The two most abundantly cloned small RNAs in the AGO2 library were miR163-LL (7.3%) and miR390 (5.6%), and those in the AGO5 library were miR163-UL (3.3%) and miR390* (1.4%) (Fig. 1C). We have previously detected miR163-LL and miR163-UL as pre-miR163-derived small RNAs (Kurihara and Watanabe 2004). Interestingly, the pair miR163-LL (abundant in the AGO2 fraction) and miR163-UL (abundant in the AGO5 fraction) form a small RNA duplex (miR163-LL/miR163-UL), and the pair miR390 (abundant in the AGO2 fraction) and miR390* (abundant in the AGO5 fraction) also form a small RNA duplex (miR390/miR390*) (Fig. 2A).

From these results, we hypothesized that each strand from these small RNA duplexes is selectively sorted for association with different AGO proteins. To confirm this, we performed Northern blot analyses with the immunoprecipitated fractions prepared from inflorescent tissues of HA-AGO2- and HA-AGO5-transgenic *Arabidopsis*. As expected, we detected miR163-LL and miR390 in the AGO2 IP fraction but not in the AGO5 IP fraction (Fig. 2B, C). Conversely, we detected miR163-UL and miR390* in the AGO5 IP fraction but not in the AGO2 IP fraction (Fig. 2B, C). These results clearly indicate that the mechanism of guide strand selection is different between AGO2 and AGO5 (Fig. 2E). Note that the accumulation of miR390 in the *ago2* mutant, which also associates with AGO4 (Qi et al. 2006), is identical to that in Col-0 (Supplementary Fig. S3), and miR163-LL and miR163-UL were detected similarly in Col-0, *ago2* and *ago5* (Supplementary Fig. S4), probably because *Arabidopsis* has AGO proteins containing redundant functions with AGO2 and AGO5.

Considering the results of Northern analysis (Fig. 2B, C) and the preference for the 5′ nucleotide in sequence analysis (Fig. 1B) together, we hypothesized that AGO2 and AGO5 select the guide strand depending on the first 5′ nucleotide (Fig. 2E); adenine for AGO2 and cytosine for AGO5. To test this hypothesis, we performed an *Agrobacterium* infiltration assay in leaves of *Nicotiana benthamiana*, because the *N. benthamiana*-based transient assay has been used to confirm the biogenesis pathways of endogenous *Arabidopsis* siRNAs including tasiRNA (Allen et al. 2005) and nat-siRNA, an *Arabidopsis* endogenous siRNA generated from a pair of *cis*-antisense transcripts (Borsani et al. 2005). In this assay, we chose pre-miR163 but not pre-miR390, because miR163 is a recently evolved miRNA in *Arabidopsis* (Allen et al. 2004) and because we confirmed the absence of endogenous miR163 and miR163 precursors in *N. benthamiana* by Northern blot analysis (Fig. 3 and data not shown). miR390, on the other hand, is highly conserved among various plants (Axtell et al. 2006). Wild-type pre-miR163, pre-miR163(WT), contains miR163-LL having a 5′ adenine nucleotide and miR163-UL having a 5′ cytosine nucleotide, whereas a pre-miR163 mutant, pre-miR163(AC), contains a mutated miR163-LL having a 5′ cytosine nucleotide, named miR163-LL(5′C), and a mutated miR163-UL having a 5′ adenine nucleotide, named miR163-UL(5′A) (Fig. 3A). As shown in Fig. 3B, miR163-UL(5′A) is associated with AGO2 more than miR163-UL, and miR163-LL is associated with AGO2 more than miR163-LL(5′C), showing that the strand of the miR163-LL/miR163-UL duplex that is preferentially incorporated into AGO2 is interchangeable, due to the exchange of the 5′ nucleotide (Fig. 3B). Similarly, miR163-UL is associated with AGO5 more than miR163-UL(5′A), and miR163-LL(5′C) is associated with AGO5 more than miR163-LL, showing that the strand of the miR163-LL/miR163-UL duplex that was preferentially incorporated into AGO5 is also interchangeable, due to the exchange of the 5′ nucleotide (Fig. 3B). Considering these results together, the first 5′ nucleotide has an important role for the guide strand selection in both AGO2 and AGO5, and the mechanism for guide strand selection is different between AGO2 and AGO5 (Fig. 3C). The difference of the guide strand selection from the same small RNA duplex in AGO2 and AGO5 (Figs. 2, 3) indicates that, in *Arabidopsis*, either AGO2 or AGO5 or both AGO2 and AGO5 select the guide strand based neither on the thermodynamic stability of the end of the small RNA duplex nor on the structure of the small RNA duplex.

Finally, we tested whether virus-derived siRNAs (vi-siRNAs) can be incorporated into AGO2 and AGO5. Wild-type (Col-0), and HA-AGO2- and HA-AGO5-expressing *Arabidopsis* plants were inoculated with an RNA virus, *Cucumber mosaic virus* (CMV) S strain, which established a latent systemic infection in *Arabidopsis*

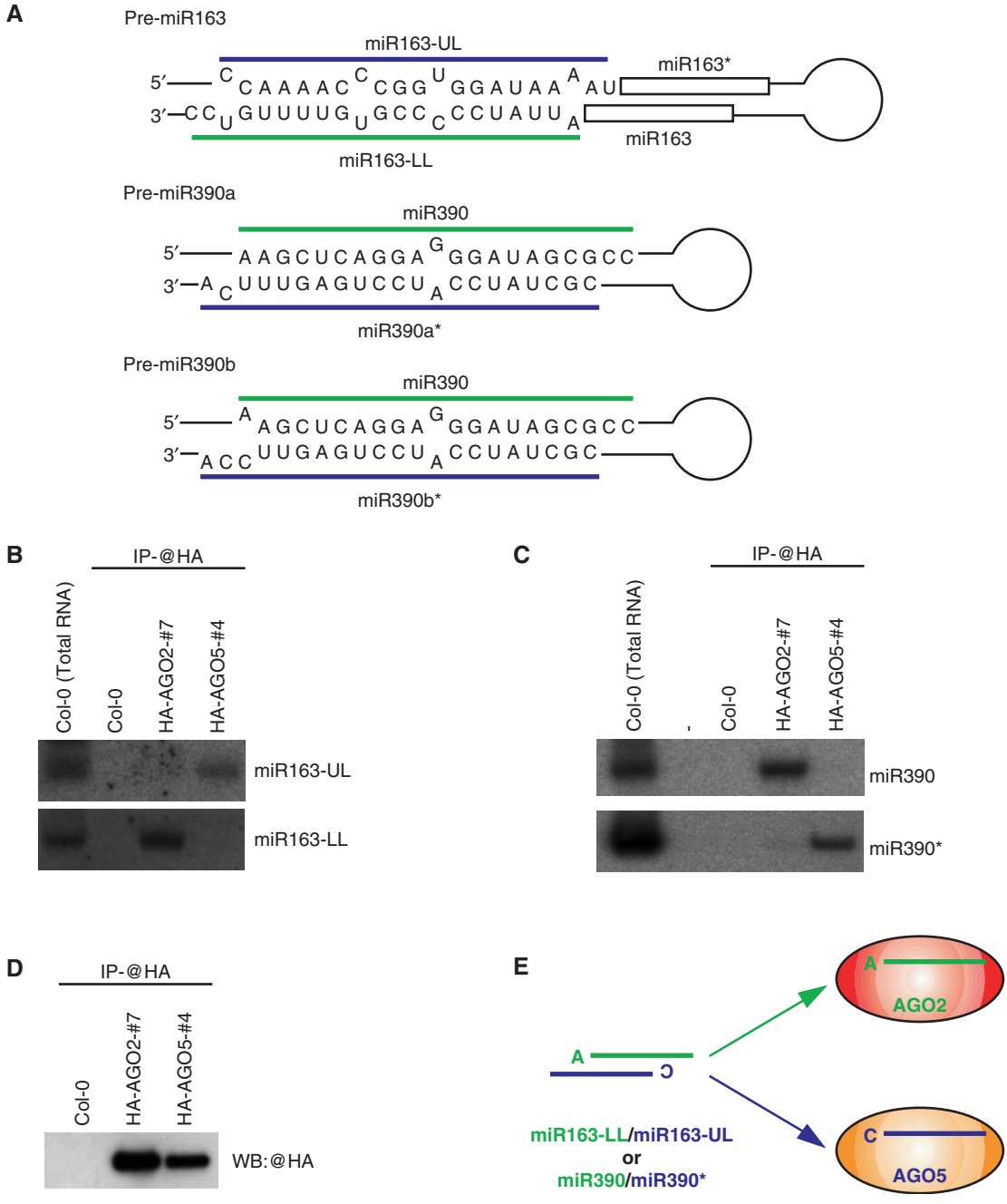

Fig. 2 Selective sorting of small RNAs between AGO2 and AGO5 in *Arabidopsis*. (A) miR163-LL/miR163-UL and miR163/miR163* in pre-mir163, miR390/miR390a* in pre-miR390a and miR390/miR390b* in pre-miR390b. (B and C) Northern blot analysis of miR163-UL and miR163-LL (B) and of miR390 and miR390* (C) using AGO2- (HA-AGO2-#7) and AGO5-associated small RNAs (HA-AGO5-#4). Col-0 is a negative control plant. We used mixed probes for miR390a* and miR390b* to detect miR390*. (D) Confirmation of HA-AGO2 and HA-AGO5 immunoprecipitation from plants by Western blot analysis with anti-HA antibody. (E) Diagram of 5′ nucleotide-dependent strand-specific association of miR163-LL, miR163-UL, miR390 and miR390* with AGO2 and AGO5.

(data not shown; kindly provided to us by Nippon Del Monte Corp.). CMV-infected inflorescent tissues were then harvested at 3 weeks post-inoculation. Northern blot analysis of CMV-derived vi-siRNAs in total RNA and in IP RNA fractions showed that both AGO2 and AGO5 have the ability to bind CMV-derived vi-siRNAs (Fig. 4). Microscopic analysis showed that both AGO2 and AGO5 localize not only in the nucleus but also in the cytoplasm (Supplmentary Fig. S5). These localization patterns support the indication that AGO2 and AGO5 can associate with

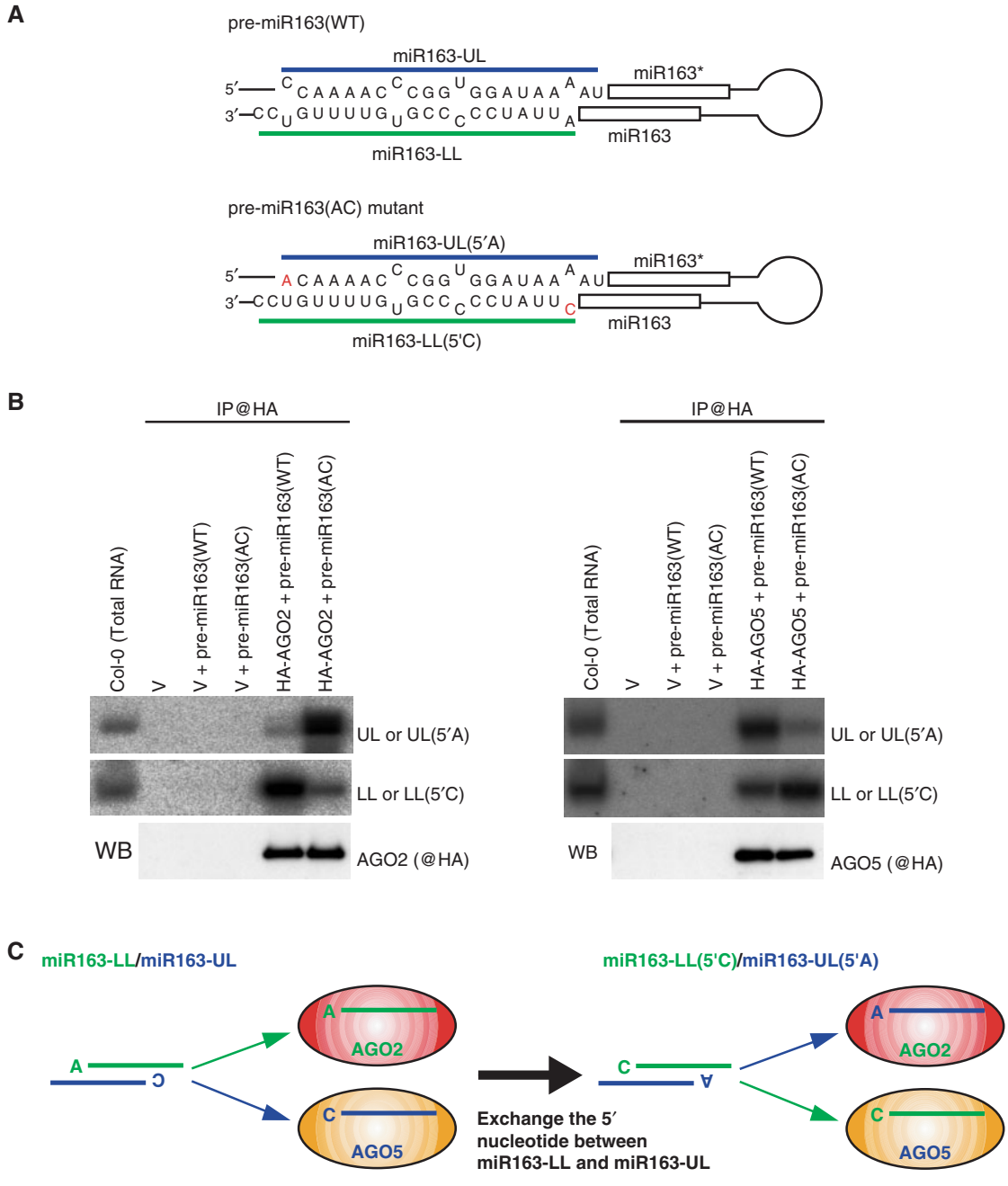

Fig. 3 Sorting into AGO2 and AGO5 is largely dependent on the first 5′ nucleotide. (A) Schematic representation of wild-type pre-miR163 [pre-miR163(WT)] and a mutant pre-miR163 [pre-miR163(AC)] used in a transient assay. (B) Northern blot analysis of miR163-UL and miR163-UL(5′A) (top rows) and of miR163-LL and miR163-LL(5′C) (middle rows) in HA-AGO2 (left panel) or HA-AGO5 (right panel) immunoprecipitation fraction. HA-AGO2 or HA-AGO5 construct, or vector only was infiltrated with pre-miR163 either (WT) or (AC). UL, miR163-UL; UL(5′A), miR163-UL(5′A); LL, miR163-LL; and LL(5′C), miR163-LL(5′C). We used 20 nt probes having complementarity with position 2–21 of these small RNAs to compare the amount of these small RNAs associated with AGO2 and AGO5. Immunoprecipitation of HA-AGO2 (left bottom row) and HA-AGO5 (right bottom row) was confirmed by Western blot analysis (WB) with anti-HA antibody. (C) Diagram of 5′ nucleotide-dependent exchange of miR163-LL and miR163-UL between AGO2 and AGO5.

CMV-derived vi-siRNA and can target CMV RNAs, which exist in the cytoplasm. Recently, Baumberger et al. (2007) showed that the polerovirus silencing suppressor P0 causes degradation of multiple *Arabidopsis* AGO proteins containing AGO1, AGO2, AGO4, AGO5, AGO6 and AGO9. Considering our results (Fig. 4) and results from previous studies (Zhang et al. 2006, Baumberger et al. 2007) together, multiple AGO genes probably have roles in

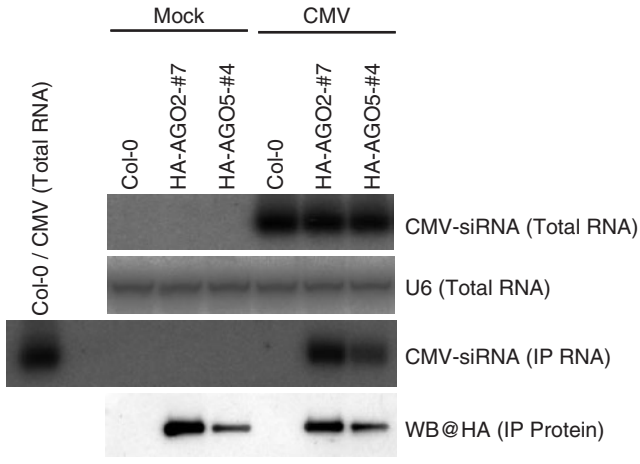

Fig. 4 CMV-derived siRNA is associated with AGO2 and AGO5. For total RNA extraction and immunoprecipitation, we used *Arabidopsis* inflorescent tissues of mock- or CMV-inoculated plants at 3 weeks post-inoculation. CMV siRNA was detected by using a CMV CP-specific random probe. U6 in the total RNA fraction is the loading control. Immunoprecipitation of HA-AGO2 and HA-AGO5 was confirmed by Western blot analysis (WB) with anti-HA antibody.

anti-virus defense in *Arabidopsis*. Based on our study (Fig. 1B), we predict that AGO1-, AGO2- and AGO5-associated vi-siRNAs also show 5′ nucleotide preference for uracil, adenine and cytosine, respectively. Considering our previous study, in which we showed that 24.8% of *Tobacco mosaic virus*-derived siRNA contains 5′ guanine (Kurihara et al. 2007), *Arabidopsis* may have an AGO gene(s) with a preference for guanine at the first 5′ nucleotide. Incorporation of the massive amount of the vi-siRNA into AGO1 seems to interfere with miRNA function, as was suggested in the case of human adenovirus VA RNA (Andersson et al. 2005), which leads to developmental abnormalities. Most *Arabidopsis* miRNAs have uracil for 5′ nucleotides (Xie et al. 2005, Fahlgren et al. 2007) and are associated with AGO1 (Qi et al. 2006). To fight against viruses with a minimum competitive influence for the AGO1–miRNA complex, plants may have acquired multiple AGO genes during evolution that have different 5′ preferences.

In this study, we presented evidence that the 5′ nucleotide of small RNA is an essential determinant to form specifically the AGO–small RNA complex in *Arabidopsis* (Figs. 2, 3). Considering our results and previous results (Khvorova et al. 2003, Schwarz et al. 2003, Steiner et al. 2007, Tomari et al. 2007), the mechanism selecting the guide strand is different among AGO proteins. Many eukaryotes have multiple AGO genes, and there may be AGO proteins that have a preference for the first 5′ nucleotide. The characteristics of the guide strand selection in most AGO proteins have not yet been examined in detail. Our results illustrate a possibility that the 'passenger strand' of siRNA duplexes, which we consider non-functional at present, may function as the 'guide strand' together with uncharacterized AGO proteins (such as miR390* in AGO5 in Fig. 2). In humans, designed 'passenger strands' of small RNA duplexes may function as 'guide strands' in AGO1, AGO2, AGO3 and/or AGO4, and may affect gene expression. The difference of off-target unexpectedly effects of siRNAs designed against the same gene and off-target effects themselves may be partly explained by action of the 'passenger strand'. We should examine the mechanism for small RNA duplex sorting between AGO proteins and for guide strand selection by each AGO protein in detail and take care to track the guide/passenger selection of designed siRNAs, especially when considering therapeutic applications.

Materials and Methods

Northern blot analysis of small RNAs

Northern blot analysis of small RNAs was performed essentially as described previously (Kurihara and Watanabe 2004). We used 30 μg of total RNA to detect ta-siR2142, simplehat2, AtREP2, siRNA02, siRNA1003, miR163-UL, miR163-LL and miR390*, 15 μg of total RNA to detect miRNAs, ta-siR255 and ta-siR1511, and 1.5 μg of total RNA to detect U6. To detect miR390*, we used mixed miR390a* and miR390b* probes (Fig. 2C). In Fig. 2B, we used a 21 nt UL probe and LL probe to detect miR163-UL and miR163-LL, respectively, whereas in Fig. 3 we used the UL20 probe and LL20 probe to compare the amount between miR163-UL and miR163-UL(5′A), and between miR163-LL and miR163-LL(5′C). These small RNAs were detected with ^{32}P-end-labeled oligonucleotide probes, which are listed in Supplementary Table S3. Radioactive signals were detected using a FUJIX BAS2500 phosphorimager (Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation of AGO2 and AGO5 from Arabidopsis inflorescent tissues

Arabidopsis inflorescent tissues containing flower stages 1–12 were ground in liquid nitrogen, and then further ground in 4 ml g^{-1} fresh inflorescent tissues of extraction buffer [20 mM Tris–HCl (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.5% Igepal-CA630, 1× complete protease inhibitor (Roche Diagnostics, Mannheim, Germany)]. Insoluble material was removed by centrifugation (5 min at 16,000 ×*g* at 4°C), and then the supernatant was filtered through a 0.22 mm filter. We then added 6 μl of an HA–agarose bead solution (3F10, Roche Diagnostics, Mannheim, Germany) per ml of the filtered supernatant, and carried out IP for 2 h at 4°C. The beads were then washed six times with 1 ml of wash buffer [20 mM Tris–HCl (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.5% Triton X-100, 1× complete protease inhibitor (Roche)]. Immunoprecipitated RNA was purified from immunoprecipitates with phenol, phenol/chloroform and chloroform extraction.

Small RNA cloning

Small RNA cloning was performed using a small RNA cloning kit (TAKARA, Tokyo, Japan) essentially according to the manufacturer's instructions. A small portion of the small RNAs after initial alkaline phosphatase treatment was used to confirm

successful immunoprecipitation. After PCR amplification of the adaptor-ligated small RNA fragment, we digested the PCR fragments with *Pst*I and ligated them with T4 DNA polymerase (NEB). We then collected ligated DNA fragments with length 300–1,000 nt using a 2% Ultra Pure agarose (Invitrogen, Carlsbad, CA, USA) gel, treated with Go Taq (Promega, Madison, WI, USA), and cloned into pCR4TOPO vector (Invitrogen, Carlsbad, CA, USA).

Sequence analysis of cloned small RNAs

We collected 18–26 nt small RNA sequences, and performed BLAST searches against the *Arabidopsis* genome, mitochondria and chloroplast sequences obtained from TAIR (ftp://ftp.arabidopsis.org/home/tair/Sequences/). The small RNA sequences that completely matched sequences in the *Arabidopsis* genome, mitochondria or chloroplast were then further annotated using the following databases: miRBase::Sequences (http://microrna.sanger.ac.uk/sequences/) for miRNA and pre-miRNA, ASRP Database (http://asrp.cgrb.oregonstate.edu/db/) for tasiRNA, MIPS Repeat Element Database (http://mips.gsf.de/proj/plant/webapp/recat/) for repeat sequences, and MIPS *Arabidopsis thaliana* Database (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp) for rRNA, tRNA, snRNA, pseudogene, cds, and putative promoter sequence covering 1,000 bp upstream of predicted and validated open reading frames. One nucleotide mismatch was allowed for the annotation shown in Supplementary Tables S1 and S2.

Agroinfiltration assay in N. benthamiana

Agrobacterium infiltration using *Agrobacterium tumefaciens* GV3101 and wild-type *N. benthamiana* was performed as described previously (Takeda et al. 2002). The concentration of *Agrobacterium* in all infiltration experiments was normalized to 1 OD at 600 nm. In the agroinfiltration assay, the *Agrobacterium* was mixed in an equal ratio (final concentration = 0.5 OD each at 600 nm). The infiltrated leaves were harvested at 2 d post-infiltration. We used 1 g of fresh leaves to perform IP. IP from *N. benthamiana* leaves was performed as described for the IP from *Arabidopsis* inflorescent tissues.

Virus inoculation and detection of vi-siRNA

The purified CMV virion (PV-1 strain) used in this study was kindly provided by Nippon Del Monte Corp. *Arabidopsis* plants were inoculated with 250 $\mu g\,ml^{-1}$ virion solution, which was dissolved in 37.5 mM sodium phosphate buffer (pH 7.0). To amplify the CP gene fragment, we designed CY3-117F and CY3-198R primers in regions conserved between five CMV strains: Y (Pubmed core nucleotide: M57602), Fny (D10538), Pepo (D28488), CS (D28489) and I17F (Y18137). We determined the CP gene sequence by sequencing two independent clones of pCRatCMV-CP, and a homology search of CP showed that CMV PV-1 is an S strain. A PCR fragment was amplified from pCRatCMV-CP using CY3-117F and CY3-198R primers, and then the PCR fragment was gel purified. The probe for the detection of CMV CP-derived siRNA was synthesized from the PCR fragment using the BcaBEST DNA labeling kit (TAKARA) and [α-^{32}P]dCTP according to the manufacturer's instructions.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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