A fungal Argonaute interferes with RNA interference

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ABSTRACT

Small RNA (sRNA)-mediated gene silencing phenomena, exemplified by RNA interference (RNAi), require a unique class of proteins called Argonautes (AGO). An AGO protein typically forms a protein–sRNA complex that contributes to gene silencing using the loaded sRNA as a specificity determinant. Here, we show that MoAGO2, one of the three AGO genes in the fungus Pyricularia oryzae (Magnaporthe oryzae) interferes with RNAi. Gene knockout (KO) studies revealed that MoAGO1 and MoAGO3 additively or redundantly played roles in hairpin RNA- and retrotransposon (MAGGY)-triggered RNAi while, surprisingly, the KO mutants of MoAGO2 (Δmoago2) showed elevated levels of gene silencing. Consistently, transcript levels of MAGGY and mycoviruses were drastically reduced in Δmoago2, supporting the idea that MoAGO2 impeded RNAi against the parasitic elements. Deep sequencing analysis revealed that repeat- and mycovirus-derived small interfering RNAs were mainly associated with MoAGO2 and MoAGO3, and their populations were very similar based on their size distribution patterns and positional base preference. Site-directed mutagenesis studies indicated that sRNA binding but not slicer activity of MoAGO2 was essential for the ability to diminish the efficacy of RNAi. Overall, these results suggest a possible interplay between distinct sRNA-mediated regulation pathways through a competition for sRNA.

INTRODUCTION

The Argonaute (AGO) protein family is a group of proteins that play key roles in RNA interference (RNAi) and its related pathways. Its members are defined by the presence of conserved domains such as PAZ, MID and PIWI (1). An AGO protein typically forms a protein complex carrying small RNA (sRNA) as a specificity determinant in downstream gene silencing events such as mRNA degradation, translational inhibition and/or heterochromatinization (2,3).

AGO proteins are evolutionarily conserved in the three domains of life. However, the number of AGO genes in each organism differs considerably. For example, there is 1 AGO gene in Schizosaccharomyces pombe, while there are 8 in Homo sapiens and 27 in Caenorhabditis elegans (4), suggesting that AGO-mediated gene regulating pathways could be diverse among organisms. The multiple AGO proteins in an organism mostly have distinct roles in development and/or genome defense, although there are some degrees of redundancy among members. In Arabidopsis, both AGO4 and AGO6 are involved in the RNA-directed DNA methylation (RdDM) pathway. However, recent studies have revealed that AGO4 and AGO6 are spatially differentially distributed in the nucleus, and have distinct, but cooperative functions in RdDM. In contrast, AGO1 and AGO10 compete for target microRNA (miRNA) binding (5). The competitive binding for miR166/165 between AGO1 and AGO10 is important for regulating shoot apical meristem development (6). Thus, the relationship between AGO proteins could be independent, cooperative or competitive.

A major biological role of RNAi is to control molecular parasites such as viruses and transposable elements (TEs) in the genome since they are generally regarded to be deleterious to the host cell. However, a genetic mutation caused by TE transposition can also benefit the host. In phytopathogenic fungi, overcoming host resistance often occurs due to a TE-mediated mutation at an avirulence gene that triggers the host defense response. For example, in the rice blast fungus Pyricularia oryzae (Magnaporthe oryzae), insertion of the Pot3 transposon into the promoter of the avirulence gene, AVR-Pita, resulted in gain of virulence toward rice cultivars carrying the corresponding disease resistance gene Pi-ta (7). Similarly, TE transposition into the avirulence genes ACE1 in P. oryzae and AVR2 in Cladosporium fulvum resulted in the switch from avirulence to virulence toward resistant rice and tomato cultivars, respectively.

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These examples clearly show that TEs can contribute to genome plasticity that allows rapid adaptation to a new environment. Thus, TE control in phytopathogenic fungi likely requires a subtle balance between repression and activation.

In the *P. oryzae* genome, we identified three AGO-like genes, MoAGO1, MoAGO2 and MoAGO3. Here we examined the roles of these three AGO genes in RNA silencing pathways of *P. oryzae*. The results indicate that MoAGO1 and MoAGO3 contributed to RNAi but, surprisingly, MoAGO2 interferes with RNAi in *P. oryzae*.

**MATERIALS AND METHODS**

**Construction of gene knockout *P. oryzae* mutants and their gene complementary strains**

The wheat-infecting *P. oryzae* isolate, Br48 and its transformants were cultured and maintained as described previously (10). Knockout (KO) mutants of three AGO genes (MoAGO1, MoAGO2, MoAGO3) in *P. oryzae* were constructed by a conventional gene targeting method by homologous recombination. Polymerase chain reaction (PCR) products of upstream and downstream fragments of a targeted gene were cloned into the multiple cloning site of pSilent2-hph that carries the Hygromycin resistance gene (hph) cassette (11). The resulting construct was introduced into fungal spheroplasts by a polyethylene glycol-mediated method as described previously (10). For initial screening, colonies PCR were performed with appropriate sets of primers for each gene. Primers used to construct gene disruption vectors and screening were given in Supplementary Table S1. The candidate strains were then examined by Southern blot analysis. Fungal genomic DNA was extracted using Plant Genomic DNA Extraction Miniprep System (Viogene) following the manufacturer’s instructions. Twenty micrograms of genomic DNA fragments were digested by an appropriate restriction enzyme. The digests were separated by agarose gel electrophoresis and transferred to Hybond N+ (Amershame) sequences attached with 2xFLAG were cloned into the multiple cloning site of pGT carrying the promoter of the glyceraldehyde-3-phosphate dehydrogenase (gpdA) gene, and the terminator of the TrpC gene in *Aspergillus nidulans* (14). The resulting FLAG-tagged MoAGO expression vectors were further modified by replacing the FLAG-tag with a fluorescent protein for cytological observation. Briefly, the vector sequences were amplified by inverse PCR using primers annealing to the upstream and downstream sequences of the FLAG-tag, and joined with a PCR-amplified mCherry or eGFP sequence by in-fusion cloning. The primer sequences used in plasmid construction are shown in Supplementary Table S1.

**RNA isolation and quantitative RT-PCR (qRT-PCR)**

RNA isolation and cDNA synthesis were performed as described previously with slight modifications (15). Total RNA was isolated from frozen mycelial powder using Sepa- sol RNA I Super (Nacalai Tesque). One microgram of total RNA was then subjected to cDNA synthesis using the ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo). qRT-PCR assay was carried out using FastStart SYBR Green Master (Roche Applied Science) or GeneAce SYBR qPCR Mix α (Nippon Gene) according to the manufacturer’s instructions with specific primers for targets and an internal control gene (actin; MGG_03982). The primer sequences are given in Supplementary Table S1. Fluorescence from DNA-SYBR Green complex was monitored through the PCR reaction. The level of target mRNA, relative to the mean of the reference housekeeping gene was calculated by the comparative Ct method. Each experiment was performed with three technical replicates.

**Immunoprecipitation, small RNA extraction and high-throughput sequencing**

Fungal mycelia were ground with mortar and pestle in liquid nitrogen, and transferred into lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetraacetic acid and 0.1% Triton X-100]. After mixing by vortex, cell lysate was collected by centrifugation at 12 000 × g for 5 min at 4°C, and incubated with Anti-FLAG M2 affinity agarose gel (Sigma-Aldrich) for 3 hr on rotation at 4°C. After washing with TBS [50 mM Tris-Cl (pH 7.5), 150 mM NaCl] three times, FLAG-tagged protein was eluted from the agarose gel by incubating with 150 ng/μL FLAG peptide for 30 min at 4°C. The elute was collected by centrifugation at 8000 × g for 30 s at 4°C.

For AGO-associated sRNA sequencing, sRNAs were recovered from the FLAG-immunoprecipitates by phenol-
chloroform extraction and ethanol precipitation. For construction of the input library, sRNAs were purified from total RNA by High Pure miRNA isolation kit. Indexed cDNA libraries were prepared with the NEXTflex Small RNA-Seq v3 kit for Illumina (Bioo Scientific) according to the manufacturer's instructions. The cDNA products were purified using Agencourt AMPure XP beads, and enriched with PCR to create the final double stranded cDNA library. The resulting libraries were sequenced on the MiSeq system (Illumina) using single end sequencing with a 75 cycle read length. Sequencing data were analyzed using Geneomics workbench software v10.1 (CLCbio). For sRNA mapping, the genome of the Magnaporthe oryzae strain 70–15 (release 8.0, http://www.broadinstitute.org/) was used as a reference sequence.

**In vitro target RNA cleavage (slicer) assay**

Target RNA cleavage assay was performed as described previously (16) with modifications. Slicer assays were performed in a reaction buffer containing 25 mM HEPES-KOH pH 7.5, 5 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol, 2 mM adenosine triphosphate, 0.05 mg/ml bovine serum albumin, 2 U/μl RiboLock RNase Inhibitor (Thermo Scientific). Immunoprecipitated AGO proteins were incubated with FITC-labeled 50 nM target RNA (5′-pGAAGGAGCUUUACAGAGGUUCG GCCGUCCAUACCAUGGG-3′) and control RNA (5′-pAACUCCUCUCUUCCGCGUAUCCCUCUU GAGCUUACCUUUAACAGCC-3′) in the reaction buffer for 1 h at 25°C. After reaction and following phenol:chloroform:isoamyl alcohol (25:24:1) extraction, RNAs were separated on a 12% polyacrylamide TBE–urea denaturing gel and visualized on Typhoon FLA 9500 (GE Healthcare).

To obtain the MoAGO2NeAD and MoAGO2Y619E+K623A mutants, one or two rounds of site-directed mutagenesis were carried out using KOD FX neo polymerase and a pair of primers containing the desired mutation (Supplementary Table S1). After PCR reaction, methylated DNA template of primers containing the desired mutation (Supplementary Figure S4). Protein domain analysis using InterProScan revealed that all three proteins possessed conserved domains (N-terminal, linker 1, PAZ, linker 2 and Piwi) and conserved amino acid residues (nucleic acid binding site, 5′ RNA guide strand anchoring site and enzymatic active site) (Figure 1B).

To elucidate the roles of MoAGO1, MoAGO2 and MoAGO3 in gene silencing, their KO mutants were constructed using the wheat-infecting P. oryzae isolate Br48 (Supplementary Figures S1–3). The resulting KO mutants, Δmoago1, Δmoago2 and Δmoago3, were first subjected to phenotypic analyses, including growth rate, germination, appressorium formation and infectivity. However, all the KO-mutants showed no significant phenotypic changes from the wild-type strain except slightly lower rates of appressorium formation in Δmoago1 and Δmoago3 (Supplementary Figure S4).

We then assessed silencing of the hygromycin resistance (hph) gene in the KO mutants induced by 2 different silencing vectors, pSilent2 and pSilent-MG. pSilent2 produces hairpin RNA of a target gene, and pSilent-MG triggers retrotransposon-induced gene silencing, as it carries the LTR-retrotransposon MAGGY with a cloning site in the retrotransposon-induced gene silencing, while MoAGO2 has MoAGO1 and MoAGO3 are involved in hairpin RNA- and retrotransposon-induced gene silencing while MoAGO2 has a negative impact on gene silencing.

**RESULTS**

MoAGO1 and MoAGO3 are involved in hairpin RNA- and retrotransposon-induced gene silencing while MoAGO2 has a negative impact on gene silencing

We first performed phylogenetic analysis of AGO-like genes in Ascomycota with emphasis on those in Sordariomycetes. AGO-like genes in the genomes of 13 fungal species were employed in the analysis. The number of AGO-like genes varied from 1 (A. nidulans, S. pombe) to 4 (Cryptonectria parasitica, Gaecumannomyces graminis) but most of the fungal species examined here possessed three AGO-like genes. Based on their amino acid sequences in the Piwi domain, the AGO genes were clustered into three major groups, which corresponded to the AGO1, AGO2 and AGO3 groups reported previously (62). Three AGO genes in P. oryzae were designated MoAGO1 (MGG 14873), MoAGO2 (MGG 13617) and MoAGO3 (MGG 01294) (62). Protein domain analysis using InterPro Scan revealed that all three proteins possessed conserved domains (N-terminal, linker 1, PAZ, linker 2 and Piwi) and conserved amino acid residues (nucleic acid binding site, 5′ RNA guide strand anchoring site and enzymatic active site) (Figure 1B).

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The levels of hph gene silencing in the resulting transfectants were initially evaluated by comparing growth rates on CM media with and without 400 μg/ml Hygromycin B. In the presence of hygromycin, transfectants of the wild-type strain with pSilent2-hyg and pSilent-MG-hyg showed an average reduction in growth rate of 45.1 and 42.5%, respectively (Figure 2A), suggesting that the hph gene was significantly silenced either by pSilent2-hyg or by pSilent-MG-hyg. In contrast, in the dicer KO backgrounds, almost no hygromycin-sensitive growth reduction was detected, regardless of the silencing vector, indicating that gene silencing was severely compromised in the dicer mutant. In Δmoago1 and Δmoago3, the rates of hygromycin-sensitive growth reduction by pSilent2-hyg and pSilent-MG-hyg were decreased to approximately 20%. This indicates that gene silencing in Δmoago1 and Δmoago3 was partly impaired at similar levels. Surprisingly, in the Δmoago2 transfectants, the rates of hygromycin-sensitive growth reduction were even higher (65.5 and 56.8% by pSilent2-hyg and pSilent-MG-hyg, respectively) than those in the wild-type strain. The enhanced gene silencing in Δmoago2 was restored to the wild-type level by gene complementation (Figure 2A).

These results suggest that MoAGO2 negatively affected the...
efficacy of hph gene silencing induced either by pSilent2-hyg (hairpin RNA) or by pSilent-MG-hyg (retrotransposon).

To verify the results of the hygromycin-sensitivity assay, the expression levels of the hph gene were examined by qRT-PCR in at least five independent transformants of each of the tested strains. Overall, the average levels of hph mRNA in the strains were mostly in accordance with the order of their hygromycin-resistance: ∆moago2 > wild-type > ∆moago1 (Figure 2B). These results show that MoAGO1 and MoAGO3 were involved in hairpin RNA- and retrotransposon-induced gene silencing pathways while MoAGO2 had a negative impact on the pathways.

Lack of MoAGO2 enhances gene silencing of the retrotransposon MAGGY and mycoviruses in *P. oryzae*

To examine the effects of the AGO genes on the activity of TEs, we introduced an active copy of the LTR-retrotransposon MAGGY (pMGY70-INT) into the AGO-KO mutants. For each tested strain, 5 to 10 independent transformants with MAGGY were subjected to qRT-PCR analysis. This analysis was performed 2 months after transformation because initial genomic copy numbers of MAGGY in the transformants differed significantly, but the copy numbers averaged with time by autonomous transposition of the element (19). In addition, we also examined...
transposition resulted in loss of the intron sequence in progeny copies of MAGGY. Thus, transpositional activity of MAGGY was assessed by the qPCR intron excision assay with sets of PCR primers specific to either the ‘exon’ junction or the intron internal sequence (Supplementary Figure S5). Figure 3B shows the ratio of the intronless and intron-containing MAGGY DNA fragments in the AGO-KO mutants and wild-type strain. Compared to the wild-type strain, the transposition ratio increased by $\sim 2$-fold in $\Delta$moago3 (Figure 3B), suggesting that MoAGO3 is the major AGO protein responsible for suppression of MAGGY transposition. In contrast, the transposition activity of MAGGY was significantly decreased in $\Delta$moago2. In $\Delta$moago1, the rate of transposition was not significantly different from that in the wild-type strain, although the level of MAGGY mRNA was elevated at levels similar to that in $\Delta$moago3 (Figure 3A and B). This was not surprising because it was previously reported that the rate of MAGGY transposition does not solely depend on the level of MAGGY mRNA in $P. oryzae$ (22).

In our previous RNA-seq analysis of the Br48 strain (23), we found two contigs highly homologous to mycoviral RNA-dependent RNA polymerase. Since their amino acid sequences were most closely related to the ourmiavirus-like virus recently reported in Botrytis cinerea (24), we tentatively designated them as $P. oryzae$ ourmiavirus-like virus 1 and 2 ($PoOLV1$ and $PoOLV2$). To assess the effects of the AGO genes on mycoviral multiplication, the levels of $PoOLV1$ and $PoOLV2$ RNA were measured by qRT-PCR in the AGO mutants. The $PoOLV1$ RNA level was $\sim 2$-fold higher in $\Delta$moago3 than in the wild-type strain, suggesting that MoAGO3 is the major AGO protein suppressing $PoOLV1$ multiplication (Figure 3C). In $\Delta$moago2, the levels of $PoOLV1$ and $PoOLV2$ RNA were dramatically decreased to $<1/1000$ compared to that in the wild-type strain (Figure 3C). Consistently, the level of $PoOLV2$ accumulation was elevated by the overexpression of MoAGO2. Since the impact of MoAGO2 KO on mycoviral multiplication was much stronger than that on TE transposition and hairpin RNA-induced RNAi, we then asked if the drastic reduction in viral RNA accumulation was due solely to enhanced RNAi using the KO mutant of MoDcl2, the dicer protein responsible for RNAi in vegetative mycelia of $P. oryzae$ (18). In the $\Delta$dcl2 background, $PoOLV2$ RNA accumulation was not affected by KO of the MoAGO2 gene at a statistically significant level. The level of $PoOLV1$ RNA was significantly decreased in the $\Delta$dcl2/$\Delta$moago2 mutant but only to $\sim 1/2$ compared to the parent $\Delta$dcl2 strain (Figure 3D), indicating that the enhanced silencing of mycoviruses in $\Delta$moago2 was mostly dependent on the MoDcl2-dependent RNAi pathway. Thus, $PoOLV1$ and $PoOLV2$ were highly sensitive to RNAi in the absence of MoAGO2. This might be related to the lack of viral coat protein (24) and/or possible lack of an RNA silencing suppressor in this group of mycoviruses. These results overall indicate that MoAGO3 is the major AGO protein responsible for RNAi against parasitic nucleic acids such as TEs and mycoviruses in $P. oryzae$, and that MoAGO2 diminishes the efficacy of RNAi in the wild-type strain.
Figure 3. Effects of AGO KO on the activity of TEs and mycoviruses. (A) qRT-PCR analysis of TEs (MAGGY and MGL) in AGO KO mutants. The actin gene (MGG03982) was used as an internal control. Asterisks indicate a significant difference from WT (two-tailed t-test; *P < 0.05; **P < 0.01). (B) Intron-excision assay was performed to examine the transposition activity of MAGGY in the AGO mutants. A relative ratio of intron-less or intron-containing MAGGY DNA was measured by qPCR using sets of primers specific to ‘exon’ junction or intron internal sequences. Asterisks indicate a significant difference from WT (two-tailed t-test; *P < 0.05; **P < 0.01). (C) qRT-PCR analysis of mycoviruses (PoOLV1 and PoOLV2) in AGO KO mutants and an AGO2 overexpression (OE) strain. The actin gene was used as an internal control. Asterisks indicate a significant difference from WT (two-tailed t-test; *P < 0.05; **P < 0.01). (D) qRT-PCR analysis of mycoviruses (PoOLV1 and PoOLV2) in the wild-type, Δdcl2 and Δdcl2/Δago2 strains. The actin gene was used as an internal control. Asterisks indicate a significant difference (two-tailed t-test; **P < 0.01).

Alteration of MoAGO1 or MoAGO3 transcription is not the cause of the enhanced RNAi phenotype of Δmoago2

To test the possibility that genetic deletion of the MoAGO2 gene caused complementary upregulation of the other AGO genes, which could result in the enhanced gene silencing of Δmoago2, the transcript levels of the AGO genes in Δmoago2 and its gene complemented strain (cAgo2) were assessed by qRT-PCR. In general, MoAGO3 mRNA was most abundant and MoAGO1 mRNA was rarest in vegetative mycelia of P. oryzae (Supplementary Figure S6). No statistically significant upregulation of MoAGO1 or MoAGO3 was detected in Δmoago2 (Supplementary Figure S6). While the average transcript levels of MoAGO1 and MoAGO3 were slightly higher in Δmoago2 than those in the wild-type strain, similar levels of MoAGO1 and MoAGO3 mRNA were observed also in cAgo2 that did not show the enhanced RNAi phenotype (Figure 2A). These results suggest that enhanced RNAi in Δmoago2 was not caused by elevated transcription of the other AGO genes, and thus, that MoAGO2 could negatively regulate the RNAi pathway in P. oryzae.

sRNAs associated with AGO proteins in P. oryzae

Deep sequencing analysis was performed to characterize sRNAs associated with the three AGO proteins in P. oryzae. Each AGO protein was tagged with FLAG at the carboxyl
terminus and expressed in the wild-type strain under the control of the A. nidulans gpdA promoter. Using cell extracts from P. oryzae mycelia expressing each FLAG-tagged AGO protein (Supplementary Figure S7), sRNAs were isolated from immunoprecipitates with anti-FLAG agarose affinity gel. Since methylguanosine-capped sRNAs in P. oryzae were previously reported to range from 16 to 218 nt in length (25), we constructed cDNA libraries using TAP-treated sRNAs without gel size selection. To reduce ligation bias (26), adapters with four randomized bases at the ligation junctions were used in the library construction. As a control to ensure the quality of the cDNA libraries, we also prepared a cDNA library using sRNA collected without FLAG-immunoprecipitation.

Each sample yielded ∼2–5 million sRNA reads sized 15–51 nt after adaptor removal (Table 1). The read mapping to the P. oryzae reference genome (70–15 strain) was performed with a cut-off of 80% coverage and 90% identity since we used a wheat infecting strain (Br48) that was closely related to but clustered differently from rice-infecting strains (27). The rates of mapped reads in the cDNA libraries were 93.4–98.2% (Table 1). Most P. oryzae sRNAs in the libraries was mapped to rRNA (57.5–87.2%) and tRNA (2.0–15.9%) as reported previously in fungi (28,29) while, surprisingly, only very small portions of sRNAs (0.3–0.2%) were mapped to repeated sequences in this strain (Table 1). Regarding 5′ base preference, A and U were generally preferred (approximately 70% in all AGO-associated sRNA libraries) (Figure 4A). MoAGO2- and MoAGO3-associated sRNAs had a relatively strong preference for 5′-A (38.0%) and 5′-U (42.7%), respectively. It should be noted that the sRNA reads might contain sequences of sRNAs that were just associated with the AGO proteins in addition to ones loaded onto them.

In comparison with the sRNA library constructed without FLAG-immunoprecipitation (input-library), sequences from rRNA, repeats and mycoviruses were enriched in all the AGO-associated sRNA libraries (Table 1), suggesting that rRNA sequences in addition to common RNAi targets (repeats and viruses) were preferentially loaded on AGO proteins in P. oryzae. In contrast, sequences from tRNA, snoRNA, intron and intergenic regions were depleted in the AGO-associated sRNA libraries (Table 1). In all the libraries, more than half of the sRNA reads were less than 25 nt (Figure 4B). A strong peak was detected at 19–20 nt in the AGO-associated sRNA libraries that likely contained small interfering RNAs (siRNAs), but it was not detected in the input-library (Figure 4B). Instead, the input-library had a sharp peak at 17 nt that was not detected in the AGO-associated sRNA libraries (Table 1). In all the libraries, more than half of the sRNA reads were less than 25 nt (Figure 4B). A strong peak was detected at 19–20 nt in the AGO-associated sRNA libraries that likely contained small interfering RNAs (siRNAs), but it was not detected in the input-library (Figure 4B). Instead, the input-library had a sharp peak at 17 nt that was not detected in the AGO-associated sRNA libraries. Almost all the 17 nt reads were mapped to several tRNA sequences, especially at their 5′ and 3′ ends. This fulfilled the feature of tRNA-derived RNA fragments (tRFs), which was originally reported in human cancer cell lines (30), and also in P. oryzae (28).

To characterize sRNA populations in different sizes, rates of 5′-U bases were examined (Figure 4C). Strong preference for 5′-U was observed in AGO-, especially MoAGO2- and MoAGO3-associated sRNA at 19–20 nt. Several other peaks, which in some cases corresponded to peaks in Figure 4B, were also detected (Figure 4C). These peaks were mostly formed due to the presence of certain abundant...
rRNA sequences at the sizes, which could correspond to small rDNA-derived RNAs (srRNAs) reported in other eukaryotes (31,32). The abundant rRNA fragments were almost all derived from transcribed regions of 26S, 18S and 5.8S rRNAs in sense orientation, and often from their partial sequences, forming a stem-like structure as shown in Supplementary Figure S8, or their 5′ and 3′ ends. Table 2 shows the 15 most abundant rRNA fragments that exhibited more than 100-fold enrichment in either of the AGO-associated srRNA libraries relative to the input-library. Interestingly, the abundant rRNA fragments were sometimes preferentially associated with a specific AGO protein in *P. oryzae*. For instance, a peak at 30–31 nt in the MoAGO1- and MoAGO3-libraries was mainly composed of 3′ end fragments of 5.8S rRNA, and a negative peak at 26 nt in the MoAGO2-library was formed due to two major non-5′-U fragments derived from a specific region in 26S rRNA (Table 2). These data clearly indicate that rRNA fragments were specifically associated with AGO protein(s) in *P. oryzae*.

In comparison among the AGO-associated srRNA libraries, rRNA sequences were relatively enriched in the MoAGO3-library and depleted in the MoAGO2-library (Table 1). srRNAs of common RNAi targets (repeats and viruses) were preferentially associated with MoAGO3 and MoAGO2 rather than MoAGO1. To further examine the genomic regions producing srRNAs associated with each AGO protein, the *P. oryzae* reference genomic sequence, which does not include the rRNA cluster and mitochondria sequences, was divided into 100-nt blocks and the number of mapped srRNAs in each block was counted. As a result, 410,272 genome blocks were made, and a total of 2,068,085 reads from the MoAGO1 (662,824 reads)-, MoAGO2 (806,874 reads)- and MoAGO3 (598,387 reads)-libraries were mapped. To reduce background noise, we focused on blocks having more than 10 mapped reads per million reads (RPM) in at least 1 library. Only 3627 (0.88%) of the 410,272 genome blocks met this criteria but included 92.8% of the mapped reads. The 3627 blocks were further classified by the number of RPMs in each AGO library. Figure 4D shows that 909 of the 3627 blocks had more than 10 RPM in every library. Surprisingly, the 909 genome blocks (0.2% of the genome) contained 89.2% of the mapped srRNA reads, indicating that AGO-associated srRNAs were derived from only very limited regions in the genome and that these srRNAs from the ‘hot spots’ were associated with every AGO protein, indicating that MoAGO1-, MoAGO2- and MoAGO3-associated srRNAs largely shared their genomic origins. However, each AGO protein also had their own mapped sites in the genome (Figure 4D) even though average number of reads per site was significantly lower than the ones at the common ‘hot spots’. Notably, MoAGO3 had relatively few specific mapped sites in the genome.

**MoAGO2 competes with other *P. oryzae* AGO proteins to bind srRNAs derived from parasitic nucleic acids**

To address the negative effects of MoAGO2 on gene silencing, we focused on srRNAs of the TEs (MAGGY and MGL) and mycoviruses (PoOLV1 and PoOLV2) (Figure 3). MAGGY- and MoOLV1-derived srRNAs were preferentially associated with MoAGO2 and MoAGO3 (Figure 5A), suggesting their competitive relationship in binding to those srRNAs. In contrast, the relative rate of MGL-derived srRNAs was much lower in the MoAGO2-library than in the MoAGO3-library, indicating that relatively less MGL-derived srRNAs were associated with MoAGO2. This may partly explain why the absence of MoAGO2 did not significantly affect the transcript level of MGL (Figure 3). Most of the TE and mycoviral srRNAs were 19–24 nt in size (Figure 5B), suggesting that they are srRNAs functioning in the RNAi pathway. Interestingly, TE and mycoviral srRNAs were peaked at 20 nt while a peak of possible srRNAs was observed at 19 nt in the original AGO-associated srRNA libraries (Figure 4B). An additional weak peak was detected at 24 nt in the TE and mycoviral srRNAs associated with MoAGO2 and MoAGO3 but not MoAGO1, suggesting that the TE and mycoviral srRNA populations associated with MoAGO2 and MoAGO3 were more closely related.

Since MoAGO2- and MoAGO3-associated srRNAs had different preferences for the 5′ base (Figure 4A), we examined 5′ and 3′ base preference in the MoAGO2- and MoAGO3-associated TE and mycoviral srRNAs. The frequencies of 5′-U and 5′-A were, however, similar between the MoAGO2- and MoAGO3-associated srRNA populations as they were for TEs and mycoviruses (Figure 5C). This was also true for 3′ base preference. These results again suggest that similar TE- and mycovirus-derived srRNA populations were competitively loaded on MoAGO2 and MoAGO3, and thus, that the absence of the competitor MoAGO2 could result in enhanced MoAGO3-mediated RNAi against the elements.

**sRNA binding but not slicer activity of MoAGO2 is essential for its ability to interfere with RNAi**

To gain further insight into the mechanism by which MoAGO2 downmodulates RNAi, we examined if MoAGO2 was an active ‘slicer’, which is an enzyme that cleaves target RNAs complementary to the bound srRNAs. Crystal structure studies indicated that the PIWI domain contains an RNase H-like fold, where the catalytic
core of the slicer enzyme is formed by four conserved residues, Asp-Glu-Asp-His (DEDH) (33–35). This signature is detectable in all P. oryzae AGO proteins as DEDH in MoAGO1 and DEDD in MoAGO2 and MoAGO3. As a negative control, we made double site-directed mutations (D689A and D763A) in the catalytic core of MoAGO2-FLAG, which was designated MoAGO2 AEAD.

FLAG-tagged MoAGO3, MoAGO2 and MoAGO2 AEAD, were purified from the cell extract of P. oryzae by immunoprecipitation and subjected to in vitro slicer assays using control and target RNAs labeled with FITC. The target RNA had a 40 nt sequence complementary to several abundant endogenous siRNA species derived from 26S rRNA. The control RNA was 50 nt in size and had a target sequence of Arabidopsis miR390 (36). By adding FLAG-tagged MoAGO3, a potential slicer in the RNAi pathway of P. oryzae, the target RNA was specifically degraded, indicating that MoAGO3 has slicer activity (Figure 6A). Surprisingly, MoAGO2-FLAG also induced specific degradation of the target RNA at a ratio (target RNA/control) similar to that for MoAGO3-FLAG. This suggests that MoAGO2 possessed slicer activity despite its negative impact on RNAi.

We next asked what characteristics of MoAGO2 are required for the ability to impede RNAi. In addition to the slicer mutant MoAGO2 AEAD, we constructed a site-directed mutant at Y619 and K623, which are supposed to be amino acid residues crucial for sRNA binding (37,38). The resulting MoAGO2 Y619E+K623A mutant showed a drastic reduction in sRNA binding (Supplementary Figure S9). The hygromycin-sensitivity assay was performed after the introduction of MoAGO2-FLAG, MoAGO2 AEAD-FLAG and MoAGO2 Y619E+K623A-FLAG into a Δmoago2 strain showing the hygromycin-hypersensitive phenotype (Supplementary Figure S10). Especially, some of the cytoplasmic granules, called P-bodies (PBs) and stress granules (SGs), both of which are evolutionally conserved ribonucleoprotein foci that contain translationally repressed mRNAs together with proteins involved in mRNA degradation and translation repression (41). Thus, the MoAGO2 and MoAGO3-associated cytoplasmic granules might be PBs or SGs in P. oryzae.

MoAGO2 and MoAGO3 are localized to the cytoplasm and cytoplasmic granules

To examine the subcellular localization patterns of MoAGO2 and MoAGO3 in P. oryzae, GFP and mCherry were fused to the C-terminus of MoAGO2 and MoAGO3, respectively. The MoAGO2-GFP and MoAGO3-mCherry fusion constructs were simultaneously introduced into the wild-type strain. mCherry and GFP signals were mainly observed in the cytoplasm, and sometimes in cytoplasmic granules (Figure 7). The pattern of the signal localization was consistent with that previously reported for AGO proteins in other organisms (39,40). The AGO proteins in various organisms were often detected in the cytoplasmic granules, called P-bodies (PBs) and/or stress granules (SGs), both of which are evolutionally conserved ribonucleoprotein foci that contain translationally repressed mRNAs together with proteins involved in mRNA degradation and translation repression (41). Thus, the MoAGO2 and MoAGO3-associated cytoplasmic granules might be PBs or SGs in P. oryzae.

Signals of MoAGO2 and MoAGO3 were sometimes co-localized but appeared to be independently distributed since the ratio of mCherry and GFP signals differed significantly depending on location in mycelia (Figure 7, merge and Supplementary Figure S10). Especially, some of the cytoplasmic granules consisted of predominantly either MoAGO2 or MoAGO3 even though the others contained both proteins almost evenly. These observations are consistent with our hypothesis that MoAGO2 and MoAGO3 can be competitive in some occasions but have roles in distinct RNA silencing pathways.
Figure 5. MoAGO2 and MoAGO3 are associated with highly similar siRNA populations derived from TEs and mycoviruses. (A) Fractions of siRNAs derived from TEs (MAGGY and MGL) and mycoviruses (PoOLV1 and PoOLV2) in the MoAGO1-, MoAGO2- and MoAGO3-sRNA libraries. (B) Size distribution of TE- and mycovirus-derived sRNA in the input (gray line)-, MoAGO1 (red line)-, MoAGO2 (yellow line)- and MoAGO3 (blue line)-libraries. (C) The relative frequency of 5’ and 3’ terminal adenine (A) and thymine (T) of siRNAs derived from TEs and mycoviruses in the input-, MoAGO2- and MoAGO3-sRNA libraries.

DISCUSSION
MoAGO2 negatively affects the RNAi (siRNA) pathway in P. oryzae

In eukaryotes, AGO proteins are involved in a variety of biological processes using sRNA as a specificity determinant. Predominant AGO-associated sRNA species include miRNA, siRNA, Piwi-interacting RNA (piRNA) and heterochromatic siRNAs (hc-siRNAs). AGO–sRNA complexes are often called RNA-induced silencing complexes (RISCs), since these complexes generally regulate gene expression negatively. For instance, miRNA-, siRNA- and piRNA-RISCs function in various types of post-transcriptional gene silencing, whereas hc-siRNA-RISCs mediate transcriptional gene silencing by inducing DNA methylation (42,43).

In filamentous fungi, mechanisms of RNA silencing have been extensively studied in Neurospora crassa, leading to identification of two major RNA-silencing pathways, namely quelling and meiotic silencing by unpaired DNA (MSUD) (44,45). Quelling is a vegetative RNA silencing while MSUD operates as meiotic silencing. Neurospora AGO genes Qde-2 and Sms-2 are involved in the quelling and MSUD pathways, respectively (44). Phylogenetic anal-
**Figure 7.** MoAGO2 and MoAGO3 are localized in the cytoplasm and cytoplasmic granules. GFP-tagged MoAGO2 and mCherry-tagged MoAGO3 were co-expressed in the wild-type *Pyricularia oryzae* cells. Images were captured using the KEYENCE BZ-9000 epifluorescent microscope and analysed using BZ-9000 software. Nuclei were visualized by DAPI staining.

In this study, the deletion of MoAGO2, one of the three *P. oryzae* AGO proteins, resulted in even higher efficiency of gene silencing than that in the wild-type when triggered by hairpin RNA, an LTR-retrotransposon, or mycoviruses. Since these triggers are typical targets of the RNAi (siRNA-mediated) pathway, MoAGO2 appeared to be a negative regulator of the pathway and thus a unique AGO protein. A possible related phenomenon is ‘sequestration’, which was reported as a competitive interaction between AGO proteins in plants, where an AGO protein acted as a decoy of a specific miRNA to sequester it from binding to the competitive AGO protein (6,46). For instance, *Arabidopsis* AGO10 competed with AGO1 for binding to miR165/166. This competitive binding diminished miR165/166-directed cleavage of the target HD-ZIP III mRNA by AGO1, and consequently regulated normal shoot apical meristem development (6). Interestingly, like MoAGO2, *Arabidopsis* AGO10 has slicer activity. However, the miRNA binding ability of AGO10 but not its slicer activity was required for sequestration (6). Similar to AGO10 in *Arabidopsis*, rice AGO18 preferentially associated with miR168 to sequester it from targeting rice AGO1 that was essential for antiviral RNAi (46). The expression of AGO18 was induced by viral infection, and subsequently led to the up-regulation of AGO1. Thus, AGO18 contributed to broad-spectrum virus resistance in rice by sequestering miR168. Our data suggested that MoAGO2 could interfere with RNAi in *P. oryzae* likely in a manner similar to that of sequestration in plants. However, competitive interaction between MoAGO2 and MoAGO3 was not limited to specific sRNA species but rather included a broad range of sRNAs derived from artificial hairpin RNA, TEs and mycoviruses.

The competitive relationship between MoAGO2 and MoAGO3 may be explained as a conflict between two distinct sRNA-mediated gene regulation pathways. In plants, siRNAs derived from various sources including hairpin RNA, TEs and viruses function in the RdDM pathway as well as in the RNAi (mRNA degradation) pathway (47,48) even though the classes of siRNAs involved in the pathways can be different at least partly. In *P. oryzae*, while no sRNA-related pathways other than RNAi have been so far identified, it may be possible that MoAGO2 is involved in RdDM or a yet unknown pathway that uses siRNA as a specificity determinant, leading to a competition for siRNA against RNAi.

Alternatively, MoAGO2 may not function as an active AGO but may act as a modulator of RNAi. Since several fungal species such as *N. crassa* do not possess an AGO family protein, this class of AGO genes may not play an essential role in fungal growth and development. Actually, Δmoago2 did not show any apparent phenotype other than enhanced gene silencing in this study (Supplementary Figure S4). As stated above, in pathogenic microorganisms, TEs sometimes benefit hosts by providing genetic alterations to adapt a new environmental condition, especially to adapt a new host. It has been observed in *P. oryzae* that a new strain overcoming host disease resistance occurred by a TE-mediated genetic change. However, in Br48, the wheat-infecting *P. oryzae* strain used in this study, the activity of TEs is extremely low likely due to repeat-induced point mutation (RIP). RIP is a process that detects DNA duplications and causes genetic changes (C:G to T:A transitions) in duplicated sequences during the sexual phase of the life cycle (49,50). Therefore, RIP can eliminate the activity of TEs permanently from the genome. Unlike common rice-infecting *P. oryzae* strains that lack the sexual phase, Br48 is highly fertile and possesses RIP as a currently active process (51). In our previous RNA-seq analysis, very few TEs were transcriptionally active (23). This was also indicated in this study, as the portion of TE-derived sRNAs was extremely low compared to that previously reported in rice-infecting *P. oryzae* strains (Table 1; (28,52)). Considering recent findings on the contributions of TEs to the genome plasticity and evolution in fungi, MoAGO2 might become a very unique modulator for maintaining TEs moderately active to generate new genetic variations in this phytopathogenic fungus.

**rRNA-related sRNAs in *P. oryzae***

Eukaryotic rRNAs such as 28S, 18S and 5.8S rRNAs are abundantly transcribed from the rDNA clusters, and make up ∼80% of the total RNA in the cytoplasm (53). In deep sequencing of sRNA, sRNAs mapped to the rRNA clusters were often omitted from detailed analysis, as they could be degradation products of abundant RNA. Surprisingly, in this study, the large majority of the AGO-associated sRNA in Br48 was derived from rRNA. This unusually high ratio of rRNA sequences in the libraries was thought to be,
at least partly, due to very low levels of repeat-derived sr-RNAs in eukaryotic cells. Nevertheless, our data clearly demonstrate that rRNA-derived srRNAs were preferentially associated with the AGO proteins in P. oryzae. Notably, some of them showed more than 1000-fold enrichment in the AGO-associated srRNA library compared to the input-library (Table 2). Thus, the association is highly specific, and not likely to depend on random degradation of rRNA.

Such abundant srRNA fragments (srRNAs) have recently been reported in mammals and plants (31,32). The characteristics of the srRNA fragments identified in this study were well matched to those of srRNAs: (i) derived from mature rRNA molecules in sense orientation, (ii) often mapped to 5’ and 3’ ends of rRNAs and internal sequences that potentially form a stem-like structure. Thus, srRNAs seem to be conserved in a wide range of eukaryotes including mammals, plants and fungi. In the fungus N. crassa, another type of rRNA-related srRNA, designated QDE2-interacting small RNA (qirRNA), was previously identified (54). qirRNA was induced in response to DNA damage, and played a role in suppressing protein synthesis after DNA damage. However, unlike srRNAs, qirRNAs were produced from non-transcribed regions as well as transcribed regions in the rDNA cluster almost equally in both sense and antisense orientations (54). These characteristics of qirRNA appear to be more closely related to DNA double strand break-induced small RNA rather than srRNAs (55).

The biological roles of srRNAs are yet to be elucidated. In mammals, overexpression or inhibition of several srRNA species led to altered expression levels of genes involved in biological processes such as glucose metabolism and cell division, but did not significantly change RNA levels (31). In this context, human AGO2 has been recently demonstrated to bind to nuclear nascent rRNA in a miRNA-mediated manner (56). PAR-CLIP-seq followed by RIP-qPCR verification identified 479 possible AGO2 binding sites in 45S rRNA, the precursor RNA that is processed into the 18S, 5.8S and 28S srRNAs. Knockdown of AGO2 resulted in a slight, but statistically significant increase in the overall rRNA synthesis (56). In addition, DICER has been shown to reside within the nucleolus and associate with rDNA regions in mammals (57). Thus, RNAi machinery seems to play roles in various rRNA-related gene regulations in a wide range of eukaryotes, with possible involvement of srRNAs.

5’ terminal bases of srRNAs are unlikely to be determinants of their association with a specific AGO protein in P. oryzae

In Arabidopsis, it was proposed that sorting of srRNAs for specific AGO loading was directed by recognition of their 5’ nucleotides (58–60). For example, Arabidopsis AGO1 preferentially binds miRNAs with a 5′-U, whereas AGO2 has a bias toward siRNAs with a 5′-A (58–60). In our study, while a strong bias to 5′-U was observed with siRNAs from TEs and mycoviruses and those srRNAs were preferentially associated with MoAGO2 and MoAGO3, a similar strong bias toward 5′-U was also observed in MoAGO1-associated TE and virus-derived srRNAs. In addition, such a strong 5′-U preference was not observed in srRNAs at different lengths (Figure 4B). Consistently, the 5′ base of the abundant srRNAs specifically associated with a certain AGO protein was not always constant (Table 2). Overall, it was unlikely that 5′ terminal bases of srRNAs determined a specific association with an AGO protein in P. oryzae. The strong 5′-U preference detected in AGO-associated srRNAs derived from TEs and mycoviruses might be due to the mechanism of their biogenesis such as the ping-pong model proposed for piRNA biogenesis (61).

DATA AVAILABILITY

The small RNA sequencing data have been deposited in the DDBJ Sequence Read Archive under the accession, DRA005932.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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