siRNA-dependent and -independent post-transcriptional cosuppression of the LTR-retrotransposon MAGGY in the phytopathogenic fungus *Magnaporthe oryzae*

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ABSTRACT

The LTR-retrotransposon MAGGY was introduced into naive genomes of *Magnaporthe oryzae* with different genetic backgrounds (wild-type, and *MoDcl1 [mdl1]* and *MoDcl2 [mdl2]* dicer mutants). The *MoDcl2* mutants deficient in MAGGY siRNA biogenesis generally showed greater MAGGY mRNA accumulation and more rapid increase in MAGGY copy number than did the wild-type and *MoDcl1* mutants exhibiting normal MAGGY siRNA accumulation, indicating that RNA silencing functioned as an effective defense against the invading element. Interestingly, however, regardless of genetic background, the rate of MAGGY transposition drastically decreased as its copy number in the genome increased. Notably, in the *MoDcl2* mutant, copy-number-dependent MAGGY suppression occurred without a reduction in its mRNA accumulation, and therefore by a silencing mechanism distinct from both transcriptional gene silencing and siRNA-mediated RNA silencing. This might imply that some mechanism possibly similar to post-transcriptional cosuppression of Ty1 retrotransposition in *Saccharomyces cerevisiae*, which operates regardless of the abundance of target transcript and independent of RNA silencing, would also function in *M. oryzae* that possesses the RNA silencing machinery.

INTRODUCTION

RNA silencing or RNA interference generally describes a process in which double-stranded RNA (dsRNA) induces the degradation of cognate mRNA (1). A long dsRNA precursor, either exogenously introduced into the cell or endogenously produced in the cell, is processed into 21–26 nt small interfering RNAs (siRNAs) by the action of the RNaseIII-like enzyme Dicer. The siRNAs are then incorporated into a protein complex called RISC (RNA-induced silencing complex) and function as molecular guides to target cognate mRNA for degradation. Because of its sequence-specific gene silencing nature, RNA silencing has been widely accepted as a fundamental genome defense mechanism to cope with invading viruses and endogenous transposable elements (TE) in a variety of eukaryotic organisms.

An interesting paradox regarding TE copy number in the genome is that higher eukaryotes possessing sophisticated genome defense mechanisms such as RNA silencing and DNA methylation are not always as successful in preventing TEs from proliferating as are lower eukaryotes with fewer defense mechanisms. For example, it was reported that 42% of the human genome consists of retrotransposons (2) while the genome of baker's yeast, *Saccharomyces cerevisiae*, contains only 3.1% retrotransposon sequences (3). Based on genome information, it seems that *S. cerevisiae* does not possess the RNA silencing and DNA methylation machineries that are shown to be effective for suppressing endogenous TEs in various eukaryotes. Therefore, it remains to be elucidated how TE copy number in the genome is controlled and to what extent silencing mechanisms contribute to this.

The phytopathogenic fungus *Magnaporthe oryzae* is a causal pathogen of blast disease on many gramineous plants (4,5). Taxonomically, it belongs to *Ascomycota*, as does *S. cerevisiae*. Because of the economic importance of this fungus, the *M. oryzae* genome has been decoded, and was published in 2005—the first such publication for a plant pathogenic fungus (6). In *M. oryzae*, retrotransposons represent 5.4% of the genome (6). Although *M. oryzae* possesses both RNA silencing and DNA methylation mechanisms, the portion of repeated sequences in the genome is a little larger than that in

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S. cerevisiae. The genome information indicates that a variety of TEs, such as DNA transposons, SINE, LINE and LTR-retrotransposons, exist in *M. oryzae*. One of the *M. oryzae* TEs, MAGGY, is a gypsy/Ty3-like LTR retrotransposon. Genomic Southern analysis has revealed that MAGGY is not ubiquitous in the *Magnaporthe* population. It was present in the genomes of *Magnaporthe* isolates from rice, foxtail millet and some grasses, but was not detectable in those from wheat, finger millet and crabgrass (7). However, when introduced by transformation, MAGGY can actively transpose in non-MAGGY carriers (8), providing a system to assess the copy number dynamics after its introduction into a naive genome.

An experimental difficulty associated with transposon research is the direct and accurate counting of the number of transposition events in the cell. Since transposition occurs independently in each cell, the amount of DNA with newly transposed elements is generally too small to detect in the cell population. The activity of TEs has, therefore, mostly been assessed by their transcriptional activity or indirectly by observations of phenotypic changes caused by transposition. Indeed, whether the transcriptional activity of TEs always parallels transpositional activity is a matter to be addressed. For example, transposition of Ty1 was very rare in the yeast genome despite the fact that Ty1 RNA is very abundant (0.1–0.8% of total RNA) in the cell (9,10). In this study, to overcome this problem, we monitored the copy number dynamics of MAGGY by serial isolation of fungal spheroplasts at certain time intervals after transformation. This approach enabled us to accurately examine the dynamics of the number of MAGGY transpositions, since new MAGGY inserts were shared by all of the cell population regenerated from the isolated single spheroplast and therefore detectable by genomic Southern analysis. Here we show that *M. oryzae* possesses two distinct post-transcriptional cosuppression pathways. One is siRNA-mediated RNA silencing and the other is an siRNA-independent mechanism.

MATERIALS AND METHODS

Magnaporthe oryzae strains, culture conditions and transformation

Br48, a *M. oryzae* strain isolated from wheat, *Triticum aestivum*, was used as a recipient in all experiments. Br48 possess no endogenous MAGGY element in the genome. The fungus was maintained on potato dextrose agar (PDA) for several months. For long-term storage, the fungus was cultured on barley seeds soaked with sucrose, dried and kept at 4°C as described previously (8). For DNA or RNA isolation and spheroplast preparation, *M. oryzae* was grown in CM liquid broth (0.3% casamino acids, 0.3% yeast extract, 0.5% sucrose) at 26°C. Fungal spheroplasts were prepared and transformed with the plasmid pMGY70 containing a copy of MAGGY and the selective maker plasmid pII99 carrying a geneticin resistance gene as described previously (8).

Southern and northern analysis

Fungal genomic DNA was extracted as described previously (8). Southern blot analysis was performed using the DIG DNA Labeling & Detection Kit™ (Roche applied science). Genomic DNA was digested with EcoRI and the digests were transferred to Hybond N$^+$ (Amersham biosciences) after fractionation on a TAE agarose gel. The hybridization and detection procedures were performed according to the manufacturer's instructions. A 0.69-kb EcoRI (nucleotide position [nt] 4659 in MAGGY)–SmaI (nt 5342) fragment of MAGGY (ES probe) was used as a probe in genomic Southern analysis.

Total RNA and low-molecular-weight RNA fractions were prepared as described previously (11) with slight modifications. Briefly, frozen mycelia (200 mg) were homogenized in liquid N_2, and 0.2 ml of Tri-Reagent (Sigma) were added. The homogenates were mixed with 0.2 ml chloroform–isoamyl alcohol (24:1). The mycelial debris was, then, pelleted by centrifugation at 10 000g at 4°C for 15 min. The supernatant was carefully removed and equal volume of isopropanol was added. Total RNA was recovered by centrifugation at 10 000g for 15 min at 4°C. The pellet was washed with 75% ethanol and dried. Low-molecular-weight RNAs were prepared from total RNA as described by Dalmay *et al.* (12). For mRNA detection, total RNA (20 μg) was separated on a 1.2% denaturing agarose gel. Prehybridization and hybridization were performed in ULTRAhyb™ (Ambion) at 68°C. The [^{32}P]-dCTP labeled ES probe was prepared by the random priming method. After hybridization, membranes were washed twice in 2× SSC (1× SSC; 0.15 M NaCl and 0.015 M sodium acetate) containing 0.2% SDS for 20 min at 68°C and subsequently washed twice in 0.1× SSC containing 0.2% SDS for 20 min at 68°C. For siRNAs analysis, low-molecular-weight RNA was separated on a 17.5% polyacrylamide–7 M urea gel. The hybridization was performed at 42°C overnight with Perfect-Hyb buffer (Sigma) with the [^{32}P]-dCTP labeled LTR probe (nt 1–253), and the membrane was washed twice at 50°C in 2× SSC containing 0.2% SDS. DNA oligonucleotides (30-, 24- and 20-mer) were used as molecular size markers. Equal loading of total RNA was estimated by ethidium bromide staining of rRNAs, or predominant RNAs in the small RNA fraction.

MAGGY transposition assay

The copy number of MAGGY was monitored by serial isolation of a single spheroplast from a cultured transformant, followed by propagation of the mycelia and Southern blot analysis of the mycelial DNA as described previously (13). The first population of the original MAGGY transformants was designated R0. The population that regenerated from a single spheroplast of R0 was called R1, one regenerated from R1 was called R2, and so on. To detect a copy of MAGGY in the genome as a band, genomic blot Southern analysis was performed using a combination of EcoRI digestion and the ES probe, which gives different sizes of hybridization signals depending on the position of the EcoRI site in the

genomic sequence flanking the MAGGY insertion (13). Because of the nature of retrotransposition, the copy number of the element increases by one per transposition. Thus, the number of increases of the element in a cell represents the number of transpositions that have occurred in that cell. The intensity of a band in the Southern blots can differ due to the timing of MAGGY transposition during the process. A MAGGY insert that had already appeared in the genome of the isolated single spheroplast would be detected as a strong band in the Southern blots while one that appeared during the culture of mycelia after the spheroplast isolation would not be detectable or would only give a faint band if detectable. Thus, we took account of the strong bands and some of the faint bands that were maintained in the following generations, and estimated the copy number of MAGGY in the single cell at spheroplast isolation.

Semi-quantitative RT-PCR analysis

Total RNA was isolated as described above. After treatment with RNase-free DNase followed by phenol extraction and ethanol precipitation, total RNA was reverse transcribed in a $20\,\mu l$ reaction using Superscript III (Stratagene) with the oligo $(dT)_{15}$ primer. For PCR amplification, $1\,\mu l$ of $5\times$ diluted RT mix was used as a template. The PCR reaction was carried out in $20\,\mu l$ with $0.5\,U$ of KOD plus DNA polymerase (Toyobo), $1.2\,mM$ $MgCl_2$ and $20\,pmol$ of each primer. The PCR program was as follows: 10 min at $94^\circ C$, 25–30 cycles of 45 s at $94^\circ C$, 45 s at $55^\circ C$ and 30 s at $68^\circ C$, followed by 10 min at $72^\circ C$. Sets of gene-specific primers used were as follows; MGR583-F: 5′-GGTGCCCTACCCAAACGATCG-3′, MGR583-R: 5′-CGGGGAAAGGGGGAGACCCTTGC-3′; MGSINE-F: 5′-GGAATGGCGCAGTGGTTAAG-3′, MGSINE-R: 5′-GTATTATTTAACGCCGGGCT-3′; Pot2-F: 5′-CCCGGAACGCGTGGTTGGACG-3′, Pot2-R: 5′-GAACGTGTGACGAATGAGGGGG-3′; MG-Act-F: 5′-GCGGTTACACCTTCTCTACCAC-3′, MG-Act-R: 5′-AGTCTGGATCTCCTGCTCAAAG-3′; MG-EF1-F: 5′-GTGACCACATGATCTCGGAGT-3′, MG-EF1-R: 5′-CCTTCTTACCGTCCTTGTTGAC-3′. PCR products were divided into two parts. One part was analyzed by gel electrophoresis and the other part was quantified in a 96-well plate using the PicoGreen dsDNA quantitation kit (Molecular Probes) and the ARVO multilabel counter (Perkin Elmer).

RESULTS

MoDcl2 (Mdl2) dicer is solely responsible for siRNA biogenesis of the LTR-retrotransposon MAGGY in vegetative mycelia

Previously, we reported that MoDcl2 (Mdl2) but not MoDcl1 (Mdl1) was involved in the production of GFP siRNAs derived from a hairpin-GFP RNA-expressing gene cassette in vegetative mycelia (14). First, we examine the dicer protein responsible for biogenesis of MAGGY siRNAs using Dicer knock-out (KO) mutants (*MoDcl1*-KO and *MoDcll2*-KO) of the *M. oryzae* strain Br48 (14) since it was reported that siRNAs were generated by
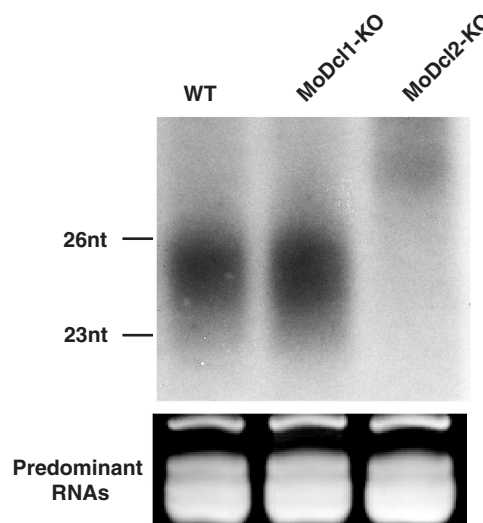
Figure 1. MoDcl2 but not MoDcl1 is responsible for MAGGY siRNA biogenesis in *M. oryzae*. Northern blot analysis of MAGGY siRNAs in *M. oryzae* transformants with different genetic backgrounds (wild-type, *MoDcl1*-KO, *MoDcl2*-KO). Small RNA fractions were prepared and separated on a 17.5% polyacrylamide gel. Equal loading of total RNA was estimated by ethidium bromide staining of predominant RNAs.

distinct dicer proteins depending on the types of triggers in some organisms such as plants (15). As shown in Figure 1, accumulation of MAGGY siRNA was detected in the wild type, indicating that MAGGY was targeted by the RNA-silencing machinery in *M. oryzae*. In the *MoDcl1*-KO mutant, accumulation of MAGGY siRNA was similar to that in the wild type. In contrast, no detectable MAGGY siRNA was found in the *MoDcl2*-KO mutant, indicating that MoDcl2 dicer is solely responsible for MAGGY siRNA biogenesis in vegetative mycelia. Those results indicated that, in *M. oryzae*, MoDcl2 dicer is likely to process a dsRNA precursor either transcribed from a hairpin-RNA-expressing gene cassette or originated from endogenous TEs. Therefore, unlike plants and *Drosophila* (16), our results failed to support the presence of distinct pathways for siRNA and repeat-associated siRNA (rasiRNA) biogenesis in *M. oryzae*.

Copy number dynamics of MAGGY after its introduction into naive genomes of *Magnaporthe oryzae* with different genetic backgrounds

We next examined the copy number dynamics of MAGGY after its introduction into naive genomes of *M. oryzae* with different genetic backgrounds (wild type, *MoDcl1*-KO and *MoDcl2*-KO). Spheroplast regenerants of the *M. oryzae* strains were serially made from R0 to R7 generations, and the copy number of the MAGGY elements in the regenerant series was monitored by Southern blot analysis (see Materials and Methods section). Five independent regenerant series, each of the wild type and the two dicer mutants were made and subjected to the assay. An example of the Southern blots is shown in Figure 2. The number of MAGGY bands in each series of regenerants was counted in the blots and plotted on a graph (Figure 3A). The average copy

numbers of the MAGGY element in the R0 populations of wild type, *MoDcl1*-KO and *MoDcl2*-KO were 10, 8.8 and 7.4, which resulted in 37.8, 37.4 and 51.6 copies, respectively, in the R7 populations. In the *MoDcl2*-KO background, the copy number of MAGGY increased
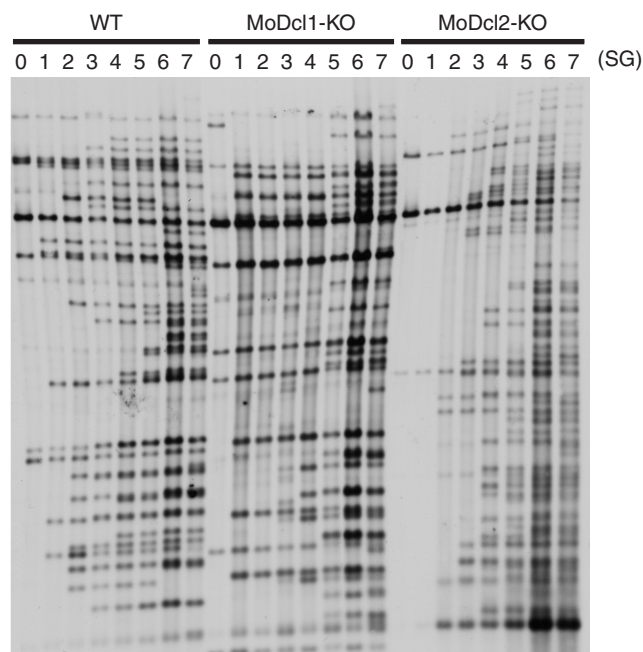

Figure 2. Southern blot analysis of the LTR-retrotransposon MAGGY in spheroplast regenerants. Copy number dynamics of MAGGY was assessed in a series of spheroplast regenerants with different genetic backgrounds (wild-type, *MoDcl1*-KO and *MoDcl2*-KO). Southern hybridization was performed as described previously (13). SG, Spheroplast generations.

more rapidly compared with the other genetic backgrounds. The difference in the average copy number of MAGGY between the *MoDcl2*-KO mutant and the wild-type or the *MoDcl1*-KO mutant was statistically significant after the R5 population by the Tukey test ($P < 0.05$). These results clearly indicated that the RNA-silencing machinery functioned as an effective defense against proliferation of MAGGY in *M. oryzae*.

Interestingly, the rate of increase in MAGGY copy number in the *MoDcl2*-KO mutants was drastically reduced after the R2 generation (Figure 3B), and similar dynamics were observed with *MoDcl1*-KO mutants and the wild type (Figure 3B). Given that the frequency of transposition of each MAGGY copy was constant, the number of MAGGY copies in the genome should increase geometrically. However, as Figure 3B shows, the transposition frequency of each MAGGY copy decreased as the population progressed. This suppression mechanism appeared to occur in response to the increase in MAGGY copy number in the genome, since the transposition rate was relatively low from the R0 generation, when the copy number of MAGGY in the population was large (see Figure 3A, wild type). It was previously shown that DNA methylation is not responsible for copy-number-dependent MAGGY suppression (13). The current results suggest that RNA silencing is also not directly involved in the suppression mechanism, since it operated even in the *MoDcl2*-KO mutant.

Cosuppression of MAGGY operates in siRNA-dependent and -independent manners

To assess the accumulation of MAGGY mRNA and siRNA during the course of MAGGY proliferation, two regeneration series, each from the wild-type and the
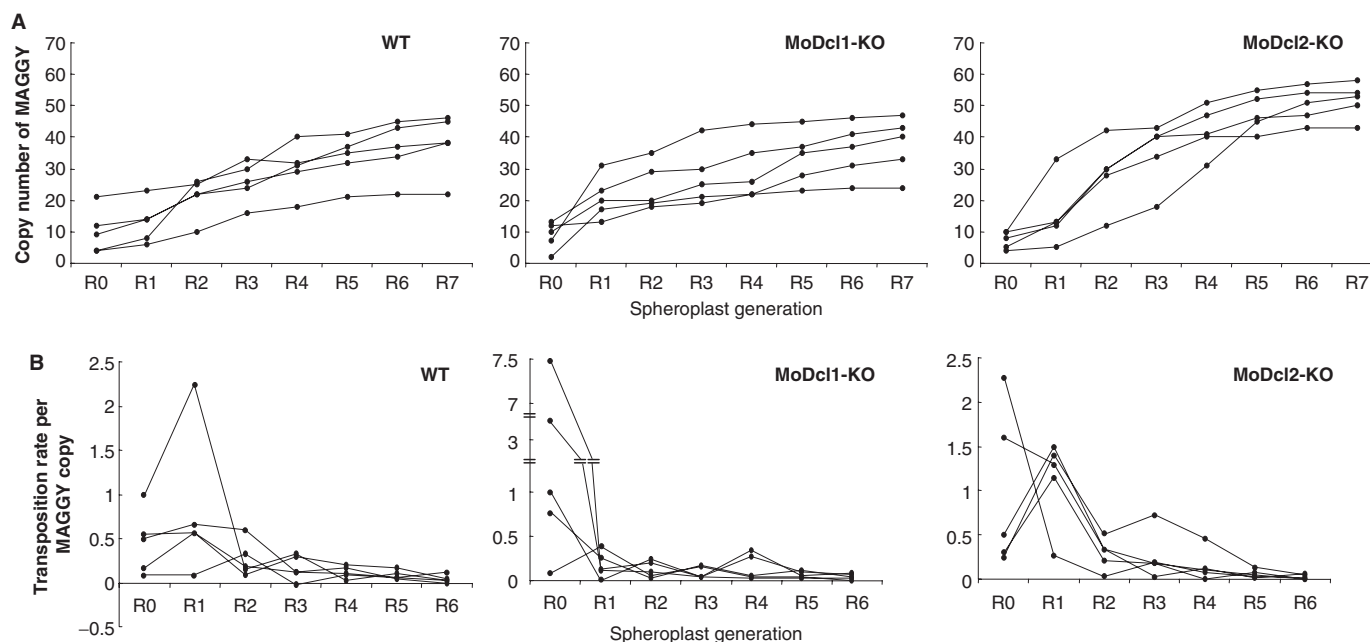
Figure 3. Copy number dynamics of MAGGY in Dicer mutants of *M. oryzae*. The copy number of MAGGY (**A**) and the rate of MAGGY transposition (**B**) in each regenerant series are shown. The rate of MAGGY transposition was calculated by dividing the copy number increment in the next generation by copy number.

A

B
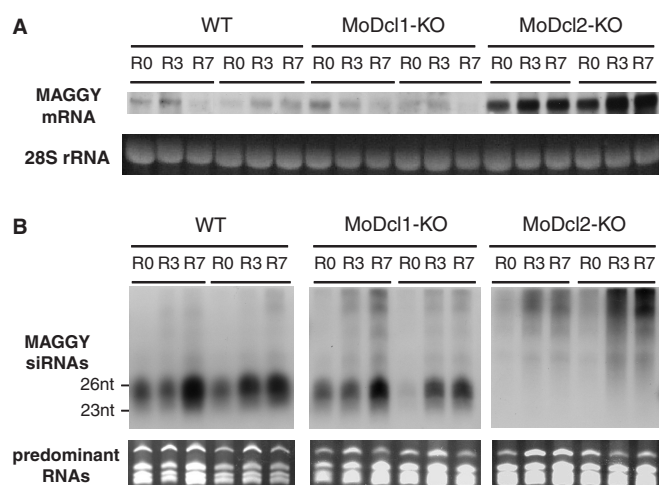
Figure 4. Copy-number-dependent MAGGY suppression in *MoDcl2*-KO mutants is not associated with transcriptional repression or mRNA degradation. Accumulation of MAGGY mRNA (**A**) and siRNAs (**B**) in the R0, R3 and R7 generations was examined by northern blots. RNA samples from two regenerant series each of wild-type, *MoDcl1*-KO and *MoDcl2*-KO were subjected to the analysis. Equal loading was estimated by ethidium bromide staining of rRNA or predominant RNAs.

MoDcl1-KO and *MoDcl2*-KO mutants were chosen randomly and RNA preparations from R0, R3 and R7 populations were subjected to northern blot analysis. MAGGY mRNA accumulation was generally much greater in the *MoDcl2*-KO mutants than in the wild-type and the *MoDcl1*-KO mutants, indicating that the RNA-silencing machinery effectively cleaved MAGGY mRNA in the wild-type and the *MoDcl1*-KO mutants (Figure 4A). In support of this, MAGGY siRNA was observed in every regenerant of the wild-type and the *MoDcl1*-KO mutants but in none of the *MoDcl2*-KO mutants (Figure 4B). Instead, in the *MoDcl2*-KO mutants, hybridizing signals of higher molecular weights were more strongly observed than in the wild-type and the *MoDcl1*-KO mutants. The higher molecular weight signals appeared to be dsRNA precursors to be processed into siRNAs by MoDcl2 protein in the normal RNA-silencing pathway in *M. oryzae*.

In the wild-type and the *MoDcl1*-KO mutant, MAGGY mRNA accumulation in the R7 populations was mostly lower than that in the R0 or R3 populations; conversely, MAGGY siRNA was more abundant in the R7 populations than in the R0 or R3 populations. These observations support the idea that RNA silencing plays a role in copy number dependent-MAGGY suppression in the wild-type and the *MoDcl1*-KO mutant. Intriguingly, there was no noticeable change in MAGGY mRNA levels among the populations in the *MoDcl2*-KO mutants, despite the fact that the transposition rate of the element was dramatically reduced as the population progressed. The absence of reduction in MAGGY mRNA accumulation indicates that MAGGY suppression is likely to operate post-transcriptionally, and thus independently of transcriptional gene silencing by chromatin remodeling mechanisms such as DNA methylation and histone modification.

It also supports the suggestion that MAGGY mRNA was not targeted for degradation by RNA silencing in the *MoDcl2*-KO mutants lacking siRNA biogenesis. These results indicate that copy-number-dependent MAGGY suppression in the *MoDcl2*-KO mutant was distinct from both transcriptional gene silencing and siRNA-mediated RNA silencing. To our knowledge, this type of TE suppression has so far not been identified in eukaryotic organisms possessing RNA-silencing machinery.

Magnaporthe oryzae endogenous TEs are not generally targeted by the RNA silencing machinery

Finally, the role of siRNA-mediated RNA silencing in the control of *M. oryzae* endogenous TEs was examined. Three representative *M. oryzae* TEs, the LINE-like element MGR583, the SINE-like element MG-SINE and the DNA transposon Pot2, were used in the analysis. In the Br48 genome, the elements were all moderately repetitive (16 to 30 copies) and appeared to be rarely active in transposition (17). Using semi-quantitative RT-PCR, transcript levels of those elements were assessed in the wild-type and the dicer mutants. Unexpectedly, there was no significant difference in transcript accumulation of any of the elements between the *MoDcl2*-KO mutant and the other genetic backgrounds (Figure 5A), indicating that none of them were targeted by siRNA-mediated RNA silencing in *M. oryzae* under normal conditions. Therefore, in *M. oryzae*, repetitive nature itself did not seem to necessarily generate a signal that triggers RNA silencing. Those results on *M. oryzae* endogenous TEs were in stark contrast to the previous northern blot analysis using the MAGGY element (Figure 4A). To examine the possibility that MAGGY was subject to RNA silencing because it was a transgene in the genome, northern blot analysis of MAGGY siRNA was performed with *M. oryzae* isolates of natural MAGGY carriers. The results indicate that MAGGY siRNA accumulation in the natural MAGGY carriers (rice isolates) was similar to that in MAGGY transformants of Br48 (non-MAGGY carrier) (Figure 5B). Therefore, MAGGY did not seem to be targeted by RNA silencing because it was introduced by transformation. We also performed northern blot analysis to examine the siRNAs of other endogenous TEs such as MGR583 and Pot2 in the natural MAGGY carriers, but the accumulation of these siRNAs was below the detectable level (data not shown). This may also support the assumption that the *M. oryzae* endogenous transposons examined here are not targeted by siRNA-mediated RNA silencing in mycelia, with the exception of the MAGGY element.

DISCUSSION

Since controlling TEs is a major constraint to the maintenance of genome integrity, multiple layers of TE suppression mechanisms are likely to function at distinct levels in eukaryote cells, including chromatin remodeling by histone modification and DNA methylation at the transcriptional level, mRNA degradation or inhibition of translation by RNA silencing at the post-transcriptional

A

B
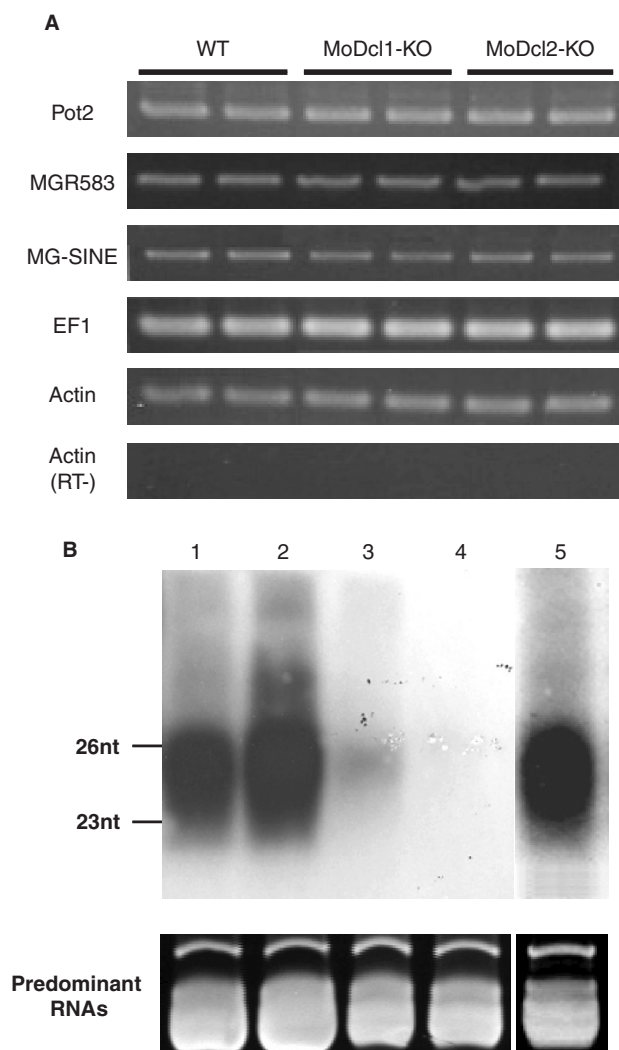
Figure 5. *M.oryzae* endogenous transposons are not generally targeted by the RNA silencing machinery. (**A**) Transcript levels of the endogenous transposons, MG-SINE, Pot2 and MGR583 were examined by semi-quantitative RT-PCR. Two independent *M. oryzae* strains each of wild-type, *MoDcl1*-KO and *MoDcl2*-KO were used. Actin and elongation factor 1a (EF1) genes were used as internal controls. (**B**) Northern blot analysis of MAGGY siRNAs in three natural MAGGY carriers of *M. oryzae* isolates from rice (lanes 1 and 2) and foxtail millet (lane 3), and one non-MAGGY carrier (wheat isolate; lane 4). A Br48 transformant with MAGGY was also subjected to the analysis (lane 5). Small RNA fractions were prepared and separated on a 17.5% polyacrylamide gel as described previously [Kadotani *et al.* (14)]. Equal loading of total RNA was estimated by ethidium bromide staining of predominant RNAs.

level, and repeat-induced point mutation (RIP) at the genetic level. Some of the mechanisms are likely to be absent in certain eukaryotes, or present in only a very limited class of organisms. RNA silencing has been shown to operate in most eukaryotes from protozoa to mammals and to play a crucial role in homology-dependent gene silencing against TEs in their genomes (18). The results of this study were fully consistent with previous reports in that the RNA-silencing machinery reduced the mRNA accumulation and transposition rate of MAGGY in *M. oryzae*. However, detailed examination of the copy

number dynamics of MAGGY in the *MoDcl2*-KO mutant indicated that *M. oryzae* possesses a post-transcriptional cosuppression mechanism that is distinct from RNA silencing because it operates independent of siRNA accumulation. This suppression mechanism is likely to belong to a new class of eukaryote host defense mechanism against TEs.

A phenomenon possibly related to the siRNA-independent post-transcriptional cosuppression in *M. oryzae* is post-transcriptional cosuppression of Ty1 retrotransposition in *S. cerevisiae* (19). *Saccharomyces cerevisiae* controls the copy number of Ty elements in the genome within a certain range, though it does not possess RNA-silencing machinery or a DNA methylation mechanism. It has been shown that homology-dependent Ty1 suppression functions at both transcriptional and post-transcriptional levels (19,20). Transcriptional cosuppression appears to be under rather transient control, switching between states in which all Ty1 elements are either transcribed or shut off. Since Ty1 RNA is generally abundant (0.1–0.8% of total RNA) in the *S. cerevisiae* cell (9,10), post-transcriptional cosuppression seems to play a more critical role in the control of Ty1 copy number in the genome. Post-transcriptional cosuppression of Ty1 retrotransposition was shown to be strongly associated with a decrease in the synthesis of Ty1 cDNA (19). Therefore, similar molecular mechanisms might be involved in the siRNA-independent post-transcriptional cosuppression of MAGGY in *M. oryzae*.

This type of cosuppression may exist only in lower eukaryotes with a small genome, such as yeasts and fungi, which are relatively successful in limiting TE proliferation. It is also possible, however, to hypothesize that siRNA-independent post-transcriptional cosuppression may be evolutionarily conserved and serves a fundamental role in the control of TE copy number in a wide range of eukaryotic organisms from budding yeast to mammals. It may be the case, as in *M. oryzae*, that siRNA-independent post-transcriptional cosuppression in higher eukaryotes is normally masked by other types of suppression mechanism such as chromatin remodeling and RNA silencing.

Recent studies have shown that TE control could have a considerable impact on genome size. The genome of *Oryza australiensis*, a wild relative of rice, has doubled in size by recent bursts of only three LTR-retrotransposon families (21). In general, eukaryotes with a small genome tend to have fewer repetitive sequences (22). Thus, an important factor in controlling genome size must be related to the suppression of TEs, especially retroelements. Given that eukaryotes have an evolutionary conserved mechanism to control genome size, this should be shared by lower eukaryotes such as *S. cerevisiae*, which has been extremely successful in keeping its genome compact. siRNA-independent post-transcriptional cosuppression may be part of the fundamental mechanisms of eukaryotes for controlling TE copy number and thereby genome size.

An alternative explanation for siRNA-independent MAGGY cosuppression can be self-regulation of transposition rate by the element itself. A large body of

empirical and theoretical studies of TEs, especially in *Drosophila*, has led to the hypothesis that the copy number of TEs is maintained as a result of the balance between transposition and natural selection (23). Hosts with heavier loads of TEs have lower fitness due to deleterious effects of insertional mutations on local genes and chromosomal rearrangements by homologous recombination between the elements, and also to costs to the host resulting from the transposition process itself such as transcription (24) and/or translation (25). Theoretical studies have shown that these factors may lead to selection for self-regulation of transposition by the element (26,27). More recently, based on a population genetic model, it has been proposed that an initial transposition burst followed by a strong limitation of its activity gives a greater chance for an invading element to 'settle down' in the host genome. (28). The copy number dynamics of MAGGY in the current study fits well in the model, and therefore theoretically can result from the strategic behavior of the element.

Indeed, with hybrid-dysgenic DNA transposons in *Drosophila*, self-regulation has been well characterized in several cases (29–33). It has been demonstrated that P-element transposition in the *Drosophila* genome was partially repressed by element-encoded repressors (34). However, in theory, the self-regulation model holds true only if there is a strong deleterious effect associated directly with the mother copy rather than its progeny copies (27). Therefore, it is not expected to occur in retroelement systems where the deleterious consequences of transposition cannot be directly linked to the mother copy (27). This theoretical prediction stands against the hypothesis that the MAGGY element has evolutionarily acquired the self-regulation mechanism. Nevertheless, we cannot rule out the possibility that MAGGY may, in fact, encode self-repressors, and therefore that siRNA-independent MAGGY cosuppression may result from self-regulation of transposition rate by the element.

In various organisms, it is often reported that a mutation in an RNA-silencing protein affects the transcript levels of different endogenous repetitive elements differently, indicating that they are not equally controlled by that RNA-silencing pathway. Similarly, in this study, accumulation of MAGGY mRNA drastically increased in the MoDcl2-KO strain whereas the transcript levels of the other TEs examined were not affected by the mutation. We assume that the differing sensitivity to siRNA-mediated RNA silencing among *M. oryzae* TEs might be associated with their transpositional activity. Previous studies showed that the numbers of possible transpositions of MAGGY, MGR583, MG-SINE and Pot2 in 24 F_1 progenies between GFSI1-7-2 (MAGGY carrier) and Br48 (non-MAGGY carrier) were 55, 1, 1 and 1, respectively, during vegetative growth (17). Therefore, the MAGGY element is likely to be much more active than the other elements in *M. oryzae*. In addition, we found that a non-autonomous MAGGY mutant deficient in the reverse transcriptase domain was not targeted by RNA silencing even when it was integrated into the genome of Br48 in multiple copies (data not shown). Thus, in *Magnaporthe*, RNA silencing may function as a counter defense against active TEs rather than a general mechanism to control the copy number of repetitive elements in the genome.

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