One of the Two Dicer-like Proteins in the Filamentous Fungi Magnaporthe oryzae Genome Is Responsible for Hairpin RNA-triggered RNA Silencing and Related Small Interfering RNA Accumulation*

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Dicer is a ribonuclease III-like enzyme playing a key role in the RNA silencing pathway. Genome sequencing projects have demonstrated that eukaryotic genomes vary in the numbers of Dicer-like (DCL) proteins from one (human) to four (Arabidopsis). Two DCL genes, MDL-1 and -2 (Magnaporthe Dicer-like-1 and -2) have been identified in the genome of the filamentous fungus Magnaporthe oryzae. Here we show that the knockout of MDL-2 drastically impaired gene silencing of enhanced green fluorescence protein by hairpin RNA and reduced related small interfering RNA (siRNA) accumulation to nondetectable levels. In contrast, mutating the other DCL, MDL-1, exhibited a gene silencing frequency similar to wild type and accumulated siRNA normally. The silencing-deficient phenotype and loss of siRNA accumulation in the mdl-2 mutant was restored by genetic complementation with the wild-type MDL-2 allele. These results indicate that only MDL-2 is responsible for siRNA production, and no functional redundancy exists between MDL-1 and MDL-2 in the RNA silencing pathway in M. oryzae. Our findings contrast with a recent report in the filamentous fungus Neurospora crassa, where two DCL proteins are redundantly involved in the RNA silencing pathway, but are similar to the results obtained in a more distantly related organism, Drosophila melanogaster, where an individual DCL protein has a distinct role in the siRNA/micro-RNA pathways.

The ribonuclease III (RNase III) family, which specifically processes double-stranded RNA (dsRNA) precursors into mature RNAs, is one of the most interestingendonuclease families. RNase III enzymes of the Dicer class are large multidomain proteins and have been shown to play an important role in the post-transcriptional gene silencing pathway triggered by double-stranded RNA. Here, we call the silencing mechanisms collectively, RNA silencing, since this phenomenon has been termed “co-suppression or PTGS” (post-transcriptional gene silencing) in plants, “quelling” in the fungus Neurospora crassa, and “RNA interference” in animals (1). In the RNA silencing pathway, Dicer cleaves long dsRNA into short RNA duplexes of 21–25 nucleotides (small interfering RNAs (siRNAs)) (2–4). Subsequently, these siRNAs are incorporated into the RNA-inducing silencing complex (RISC) to serve as sequence-specific guides for targeting cognate mRNA for degradation (5). Recently, Dicer has been implicated in downstream processes of siRNA production in the silencing pathway. Depletion of Dicer in mammalian cells resulted in a significant reduction in gene silencing efficiency induced by cognate siRNA treatment (6). Supporting this, Dicer has been shown to physically interact with protein components of RISC and siRNAs (7). In addition to the RNA silencing pathway, Dicer also plays an essential role in developmental pathways by generating regulatory 19–25-nucleotide small RNAs, termed micro-RNAs (miRNAs), from longer partially double-stranded RNA precursors (8–10). miRNAs negatively regulate gene expression by annealing to the 3′-untranslated region of target transcripts and directly interfere with translation (11, 12) or, in some cases, by inducing sequence-specific nucleolysis (13, 14). Therefore, Dicer enzymes are key components in the small RNA-mediated gene regulation mechanisms.

Genome-wide sequencing projects have revealed that Dicer-like (DCL) proteins are evolutionarily conserved in a wide range of eukaryotic genomes. Interestingly, the number of DCL proteins in the genome varies among distinct organisms (15). For example, only one DCL protein has been identified in the genomes of human, mouse, nematode Caenorhabditis elegans, and fission yeast Schizosaccharomyces pombe, whereas multiple DCL proteins have been found in other organisms such as the fruit fly Drosophila melanogaster (two), plants Arabidopsis thaliana (four) and Oryza sativa (two), and filamentous fungi N. crassa (two) and Magnaporthe oryzae (two). It is not still clear how the multiple DCL proteins in the genome participate in the siRNA/miRNA silencing pathways. Catalanotto et al. (16) recently showed that transgene-induced RNA silencing in N. crassa was not compromised in either of the single mutants of the two DCL proteins but was impaired in the double DCL mutant, thereby suggesting that both N. crassa DCL proteins were redundantly involved in the RNA silencing pathway. In contrast, Lee et al. (17) demonstrated that both of the single Dicer mutants (dicer-1 and dicer-2) in Drosophila had some degree of deficiency in the silencing phenotype induced by hairpin-RNA and indicated that both Dicer-1 and Dicer-2 were involved in the RNA silencing pathway by playing different roles; Dicer-2 was responsible for siRNA production in the initiation step, and both Dicer-1 and Dicer-2 were involved in...
Dicer-like Proteins in M. oryzae

siRNA-dependent RISC formation in the effector step. In addition, they showed that Dicer-1 was solely responsible for the production of mature miRNA. Therefore, Dicer-1 and Dicer-2 appear to have distinct roles in the siRNA/miRNA silencing pathways with respect to the small RNA species they generate. Similar to Drosophila, DCL proteins in Arabidopsis also appear to function differently in the siRNA/miRNA silencing pathways (18, 19). Mutation of DCL1 (CAF), one of the four Arabidopsis DCL proteins, blocked miRNA production but not siRNA accumulation or hairpin RNA-induced silencing, whereas those of DCL2 and DCL3 resulted in a decrease in virus-derived and endogenous siRNA biogenesis, respectively (19). One possible explanation for the difference in redundancy of DCL proteins in the RNA silencing pathway among the organisms could be that functions of DCL proteins may be differentiated during evolutionary processes from lower eukaryotes such as fungi to higher eukaryotes such as plants and insects. Alternatively, it is possible that the degree of functional diversity of DCL proteins differs even in closely related organisms.

The ascomycete fungus M. oryzae (formerly Magnaporthe grisea) (20) is the causal agent of blast disease on many graminaceous plants and is taxonomically close to N. crassa. Due to its economic importance, the genome sequence of rice blast fungus was determined in 2002 (21). Thus, M. oryzae offers a model system for elucidating fungal pathogen-plant interactions. We have demonstrated that RNA silencing occurred in the fungus with the molecular features consistent with this type of gene silencing (22). We identified two DCL-like genes, by data base comparison, in the genome of M. oryzae and tentatively named them MDL-1 and -2 (Magnaporthe Dicer-like-1 and -2). To gain insights into roles of MDL-1 and -2 in M. oryzae, we disrupted these genes by homologous recombination and examined the characteristics of the mutants in detail. Here we demonstrate that one of the two DCL proteins in M. oryzae is responsible for siRNA production in the RNA silencing pathway.

EXPERIMENTAL PROCEDURES

M. oryzae Strains and Culture Conditions—M. oryzae strain Br48 isolated from wheat, Triticum aestivum (L.) Thell, was used as a recipient in transformation. The fungus was maintained on potato dextrose agar 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 (22). For pyrithiamine screening, Czapek medium (0.05% yeast extract, 0.5% sucrose) at 26 °C.

Transformation of M. oryzae—Protoplast preparation and fungal transformation were performed as described previously (23). Three plasmids, pSH75 (24), pII99 (25), and pPTR-EGFP2, carrying the hygromycin phosphotransferase gene (HPH) cassette into exons of those genes. For pMDL1-hph targeting for the 2.1-kb fragment, 5'-GGCGGGCCGCGCAACCGGGCTGGTGACGG-3' and 5'-CCCTCTAGATCTTCCTATCCGGGAGTCTG-3' were cloned into pBluescript SKⅡ in a one-by-one manner so that two fragments maintained correct orientation and order. The HPH cassette amplified by PCR as above was then inserted in the middle of the two fragments at the EcoRV site, resulting in pMDL2-hph. The silencing construct pEGFP-SA-neo was constructed by modifying pEGFP-SA that expressed hairpin RNA of the enhanced green fluorescence protein (EGFP) gene (22). A neomycin phosphotransferase gene (NPTII) cassette was cloned into pBlueScript SKⅡ in a one-by-one manner so that two fragments maintained correct orientation and order. The HPH cassette amplified by PCR as above was then inserted in the middle of the two fragments at the EcoRV site, resulting in pMDL2-NT-RV used for genetic complementation of the md-2 mutant was constructed by inserting a 7.4-kb EcoRV-NotI fragment derived from a bond NT33H1 into pBlueScript SKⅡ-H. The cosmid NT33H1 was isolated by screening a cosmid library of M. oryzae Br48 with a 2.1-kb DNA fragment of the MDL-2 gene.

Imaging and Measurements of GFP Fluorescence—The GFP fluorescence of transformant colonies on potato dextrose agar medium was measured by the ProXpress Proteomic Imaging System and ProFinder software (PerkinElmer) as described previously (22). The GFP fluorescence was detected using 485-nm excitation and 535-nm emission wavelengths.

Southern and Northern Analysis—Fungal genomic DNA was extracted as described previously (23). Southern blot analysis was performed using the dioxetane chemiluminescence system Gene ImageTM (Amersham Biosciences). Genomic DNA was digested with an appropriate restriction enzyme(s), 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.

Total RNA and low molecular weight RNA fractions were prepared as described previously (22). Briefly, frozen mycelia were homogenized with a mortar and a pestle in liquid N2. An equal volume of 2 ml each of extraction buffer (0.5 M LiCl, 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)) and ethanol (80%) was added to the mycelia. The homogenates were mixed with 2 ml of chloroform/isooamyl alcohol (24:1). The mycelial debris was then pelleted by centrifugation at 10,000 × g at 4 °C for 20 min. The supernatant was carefully removed and extracted twice with an equal volume of chloroform/isooamyl alcohol (24:1). Total RNA was precipitated from the aqueous phase with 1 vol of 70% ethanol and washed with 70% ethanol. Low molecular weight RNAs were prepared from total RNA as described by Dalmay et al. (26). For mRNA detection, total RNA (20 μg) was separated on a 1.2% denaturing agarose gel. When dsRNA was a target for detection, total RNA was treated with S1 nuclease (Takara) at 37 °C for 3 h and precipitated with ethanol. The pellet was dissolved in 100% formamide, 6× sodium dodecyl sulfate (SDS) buffer at 42 °C for 5 min and used for hybridization. After electrophoresis, RNA was transferred to Hybond NX (Amersham Biosciences). Prehybridization and hybridization were performed in ULTRAhyb™ (Ambion) or PerfectHyb buffer (Sigma) at 68 °C.

[32P]dCTP-labeled probes were 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, 7M urea gel. After blotting onto Hybond NX, the hybridization was performed at 42 °C overnight with PerfectHyb buffer, 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 RNA bands, of predominant RNAs, or of cleaved RNAs by S1 nuclease upon their availability.

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-μl reaction containing RevertAid H Minus (Toyobo) with the following components: 4 μl of 5× RT buffer (Toyobo), 1.2 mM MgCl2, and 20 pmol of each primer. The PCR program was carried out in 20 μl with 0.5 unit of Taq DNA polymerase (Toyobo), 1.2 mM MgCl2, and 20 pmol of each primer. The PCR program was as follows: 10 min at 94 °C, 25 cycles of 45 s at 94 °C, 45 s at the appropriate annealing temperature (50–65 °C), and 45 s at 72 °C, followed by 10 min at 72 °C. Sets of gene-specific primers used were as follows: MDL1-F (5’-AGCGTACGCTGGCGTAGGTTG-3’) and MDL1-R (5’-CCCTCTAGATCTTCCTATCCGGGAGTCTG-3’).
CACGCATTCTGCTCCTGTG-3
/H11032
MDL2-F (5'-H11032-TGGACTGATCAAC-
GAACATC-3'-H11032)
MDL2-R (5'-H11032-GAAACTAGGGTCTGTACTAC-3'-H11032)
B-TUB-F (5'-H11032-TTCCCCCGTCTCCACTTCTTCATG-3'-H11032)
B-TUB-R (5'-H11032-GACGAGATCGTTCATGTTGAACTC-3'-H11032)

Sequence Analysis—Sequencing reactions were carried out using the ABI Prism Big-dye Terminator Ready Reaction sequence kit (Applied Biosystems) and analyzed with an ABI310 sequencer. The International Rice Blast Genome Consortium Magnaporthe data base (available on the World Wide Web at www.broad.mit.edu/annotation/fungi/Magnaporthe/) and BLAST program were used for data base searches. The Pfam server (available on the World Wide Web at www.sanger.ac.uk/Software/Pfam/) was used to search for conserved domains in the sequence. Multiple sequences of helicase and two RNase III domains were aligned using the ClustalW program (available on the World Wide Web at www.ebi.ac.uk/clustalw/), and phylogenetic analysis was performed.

RESULTS

Identification of two M. oryzae Dicer Homologues and Construction of Mutants—Searches of the Magnaporthe data base (available on the World Wide Web at www.broad.mit.edu/annotation/fungi/Magnaporthe/) for Dicer-like proteins resulted in the identification of two possible candidates (MG01541.4 and MG07167.4), which we designated as MDL-1 and -2 (Magnaporthe Dicer-like-1 and -2, respectively). MDL-1 encodes a protein of 1658 amino acids exhibiting three distinct characteristic domains of Dicer: an N-terminal DEAD/DEAH box helicase, two C-terminal RNase III signatures, and a domain of unknown function DUF 283. MDL2 is a protein of 1485 amino acids also displaying the characteristic domains of Dicer, but the DUF 283 domain is weakly conserved in MDL2 (Fig. 1). In addition, both MDL1 and MDL2 harbor possible dsRNA binding domains at the C terminus, which show recognizable similarity to dsRNA binding domains found in other DCL proteins. Cluster analysis of conserved helicase and RNase III domains in DCL proteins revealed that MDL1 and MDL2 were most closely related to DCL1 and DCL2 in N. crassa, respectively, among known DCL proteins (data not shown).

To examine the roles of MDL1 and MDL2 in the RNA silencing pathway in vivo, mutations in each gene were constructed by homologous recombination. Two vectors containing HPH gene cassette insertions in the exons of either the MDL-1 or MDL-2 genes were constructed. Each vector was then transformed into the M. oryzae isolate Br48. Transformants were screened by PCR analysis for homologous recombination of the HPH cassette into either mdl-1 or mdl-2 (data not shown) and further analyzed by Southern blotting. Southern blots (Fig. 2, A and B) showed the integration of the vectors at the expected site. Two mutants each of MDL-1 (mdl1–4 and -10) and MDL-2 (mdl2–17 and mdl-36) were obtained. The mutants mdl1–4 and

FIG. 1. Schematic representation of MDL1 and MDL2 proteins in M. oryzae. The boxes indicate protein domains conserved in Dicer-like proteins. HELN, DExH box-like domain; HELC, a helicase C domain; DUF283, conserved domain with unknown function; RNa, RNase IIIa domain; RNb, RNase IIIb domain; DRB, dsRNA binding domain.

FIG. 2. Disruption of the MDL-1 and MDL-2 genes by homologous recombination in M. oryzae. Southern blot analysis of mdl-1 and mdl-2 disruptants. EcoRI and SmaI were used for genomic DNA digestion of mdl-1 disruptants (A), and XhoI was used for that of mdl-2 disruptants (B). The digests were fractionated on a 0.7% TAE-agarose gel and probed with the DNA fragments indicated in the schematic depictions of the MDL-1 and MDL-2 loci with or without the disruption vectors. C, RT-PCR analysis of MDL1 and MDL2 mRNA expression in the mdl-1 and mdl-2 disruptants. Total RNA was reverse-transcribed with an oligo(dT) primer and used as a template for RT-PCR analysis with sets of specific primers. Specific primers for the β-tubulin gene were used to detect constitutively expressed mRNA. Total RNA without reverse transcription was used as a negative control. WT, wild type.
mdll-1 and mdl-2 were primarily used in further analyses and were designated as mdl-1 and mdl-2 mutants, respectively.

To confirm that the disrupted genes in both mutants were not functional, expression of the MDL-1 and -2 genes was examined by RT-PCR using specific sets of primers as described under “Experimental Procedures.” The β-tubulin gene was used as an internal control. Fig. 2C showed MDL2 mRNA but not MDL1 mRNA in mdl-1, and MDL1 mRNA but not MDL2 mRNA in mdl-2 were detectable in this assay, indicating the loss of function of the MDL-1 gene in the mdl-1 mutant and of the MDL-2 gene in the mdl-2 mutant.

To examine the effect of MDL1 and MDL2 on vegetative growth, colony growth assay was performed. Wild-type and mdl mutants were cultured on potato dextrose agar medium without antibiotics for 5 days in the absence of light, and colony sizes were subsequently measured. Slightly slower growth was observed with the mdl-2 mutant compared with wild-type at 22 and 30 °C but not at 20 °C (Table I). The growth rate of the mdl-1 mutant was equivalent to that of wild-type at any temperature examined.

Disruption of MDL-2 but not MDL-1 Impaired RNA Silencing in M. oryzae—Gene silencing assays of the mdl mutants were performed by two steps of transformation. First, test strains were transformed with pEGF75 carrying the EGFP gene under control of the Aspergillus nidulans trpC promoter. After screening the resulting transformants for strong GFP fluorescence, pEGFP-SA-neo, carrying transcriptional units for the neomycin phosphotransferase gene as a selectable marker and for hairpin RNA of the EGFP gene was introduced into the GFP-expressing transformants. After selection by antibiotics, double transformants containing pEGF75 and pEGFP-SA-neo were randomly selected, and their GFP fluorescence was measured using the image analyzer ProExpress (Applied Biosystems). Based on the intensity of GFP fluorescence, the double transformants were categorized into five classes, and the number in each class was represented in a graph (Fig. 3A). In the mdl-1 double transformants, loss of GFP fluorescence was observed at a frequency comparable with that in wild type. In contrast, loss of GFP fluorescence was barely detected in the mdl-2 double transformants. These results suggested that MDL2 but not MDL1 was involved in the RNA silencing pathway. However, there were some contradictory results (e.g. strong GFP fluorescence was observed in some mdl-1 double transformants and also in the wild-type strain). In addition, a few mdl-2 transformants also showed a silenced phenotype. To address this, silenced (S in Fig. 3) and nonsilenced (N in Fig. 3) transformants were selected from each of the mdl-1, mdl-2, and wild-type strains, and Southern analysis was used to examine the integrated vectors pEGF75 and pEGFP-SA-neo. Double digestion of the vectors with EcoRI and XbaI should give rise to 2.6- or 3.2-kb fragments containing transcriptional units of EGFP mRNA and hairpin RNA in pEGF75 and pEGFP-SA-neo, respectively. Southern blots with EcoRI- and XbaI-digested genomic DNA and an EGFP probe indicated that the transcriptional units of both EGFP hairpin RNA and mRNA remained intact in the silenced transformants of mdl-1 and wild type. In the nonsilenced transformants, the 3.2-kb band corresponding to the intact transcription unit of EGFP hairpin RNA was not detected, indicating that integration of pEGFP-SA-neo might have occurred by interruption of a transcriptional unit or that some rearrangement might have occurred after the integration event. The 3.2-kb band was not present in all nonsilenced mdl-1 transformants examined. Those results indicated that the mdl-1 mutant have no phenotypic deficiency in the RNA silencing pathway triggered by hairpin RNA.

In the mdl-2 mutant, the 2.6- and 3.2-kb fragments were detected in most of the nonsilenced transformants (Fig. 3B), indicating that the nonsilenced phenotype of the transformants was not due to the loss of the transcription units of EGFP hairpin RNA but probably due to a deficiency in the RNA silencing pathway. One double transformant of the mdl-2 mutant showed a complete silenced phenotype (Fig. 3A). Southern analysis revealed that the 2.6-kb band corresponding to the transcription unit of EGFP mRNA was absent in the transformant. Therefore, it was likely that the silenced phenotype of the transformant was simply attributed to the loss of the EGFP gene but not to RNA silencing. In conclusion, the results obtained here indicated that mutation of MDL-2 but not MDL-1 impaired RNA silencing triggered by hairpin RNA in M. oryzae.

**MDL-2 Is Essential for dsRNA Dicing**—To determine the biochemical roles of MDL1 and MDL2 in the RNA silencing pathway, siRNA production and mRNA accumulation were examined by Northern analyses. GFP-expressing transformants with or without the intact silencing construct pEGFP-SA-neo were employed with different genetic backgrounds of wild-type, mdl-1, and mdl-2. Accumulation of EGFP siRNAs was induced by introducing the silencing construct in the wild-type and mdl-1 transformants and not in the mdl-2 transformants, consistent with the idea that MDL2 processed dsRNA into siRNAs in M. oryzae (Fig. 4A). Note, no siRNA accumulation was detectable in the mdl-2 mutant even when the blot was overexposed (Fig. 4A). This is in contrast to previous reports on DCL mutants in other organisms, where a low level of siRNA accumulation was detectable even in mutants of DCL proteins that were mainly responsible for siRNA production (16–17, 19).

As with the previous phenotypic assay, a drastic decrease in EGFP mRNA accumulation by the silencing construct was observed with wild-type and mdl-1 backgrounds (Fig. 4B). However, in the mdl-2 transformant, no decrease in EGFP mRNA accumulation was observed by the silencing construct. This probably was due to the lack of EGFP siRNA accumulation in the mdl-2 transformant, which led to loss of a sequence-specific guide for EGFP mRNA degradation in the RISC complex.

To confirm that the absence of EGFP siRNAs in the mdl-2 transformant was attributed to a deficiency in the dicing process of hairpin RNA but not due to inhibition of hairpin RNA expression, further Northern analysis was performed to detect a double-stranded form of EGFP RNA with S1 nuclease treatment. Fig. 4C showed that dsRNA of EGFP was detected only in transformants containing the silencing construct. These results strongly indicated that the mdl-2 mutant had a deficiency in the dicing process of the RNA silencing pathway.

**Complementation of the mdl-2 Mutant with the Wild-type MDL-2 Gene Restored RNA Silencing and siRNA Accumulation in the Mutant**—To directly demonstrate that the mdl-2 mutation was responsible for the deficiency in RNA silencing and siRNA accumulation in the mutant, genetic complementation was performed. A cosmid library of wild-type M. oryzae (Br48) was screened using a MDL-2 probe, and three cosmids were isolated. Restriction analyses of the cosmids with reference to

### Table I

| Strain         | Colony size at 20 °C | Colony size at 22 °C | Colony size at 30 °C |
|----------------|----------------------|----------------------|----------------------|
| Wild type      | 1.77 ± 0.08          | 2.09 ± 0.09          | 3.10 ± 0.07          |
| mdl-1          | 1.71 ± 0.05          | 2.09 ± 0.11          | 3.16 ± 0.08          |
| mdl-2          | 1.69 ± 0.11          | 1.88 ± 0.13          | 2.94 ± 0.13          |

* Significant difference by Student’s t test (1%).
the *M. grisea* genome data base allowed us to identify a 7.4-kb NotI-EcoRV fragment containing the entire *MDL-2* gene. A *mdl-2* mutant expressing GFP fluorescence (*mdl*-2-36-g1) was transformed with the silencing vector pEGFP-SA-neo carrying a transcription unit for hairpin RNA of the EGFP gene. One hundred fifty transformants were picked out from each transformation, and their GFP fluorescence was measured with the imaging system described under “Experimental Procedures.” The transformants were then classified into five categories (0–20, 21–40, 41–60, 61–80, and above 81%) based on fluorescence relative to the corresponding parent recipient strain. The number of double transformants in each category was plotted in the graph. The asterisk indicates a *mdl-2* transformant with silenced phenotype that is further analyzed by Southern blots in B. B, Southern blot analysis of the *M. oryzae* transformants with silenced or nonsilenced phenotype under different genetic backgrounds. Genomic DNA was digested with EcoRI and XbaI, fractionated on a 0.7% TAE-agarose gel, and probed with the EGFP gene. The open triangle indicates the 2.6-kb fragment corresponding to the transcriptional unit of EGFP mRNA, and the closed triangle represents the 3.2-kb fragment in pEGFP-SA-neo containing the transcriptional unit for hairpin RNA of the EGFP gene. S, silenced transformants; N, nonsilenced transformants.

**DISCUSSION**

Do Multiple DCL Proteins Function Redundantly in the siRNA-mediated Gene Silencing Pathway Triggered by Hairpin RNA?—Small RNAs are now regarded as sequence-specific guides in a variety of gene regulation processes including posttranscriptional mRNA degradation, translation inhibition, and modification of chromatin structure. Small RNAs are processed from longer RNA precursors with double-stranded features by the action of the Dicer class RNase III like enzymes. DCL proteins are conserved in a wide range of eukaryotic genomes in single or multiple copies (15). In this study, we demonstrated that siRNAs involved in the RNA silencing pathway were produced by MDL2 in *M. oryzae*, one of the two DCL proteins in the genome. No redundancy in siRNA production between the DCL proteins was detected. Therefore, the two *M. oryzae* DCL proteins have distinct functions in small RNA-mediated gene regulation mechanisms. By analyzing ethyl methane sulfonate-induced mutants, Lee et al. (17) recently assigned distinct roles to the two dicer proteins.

**FIG. 3.** *mdl-2* but not *mdl-1* disruptants were deficient in RNA silencing. A, GFP-expressing recipient strains with different genetic backgrounds (*mdl-1, mdl-2,* and wild type (WT)) were transformed with the silencing vector pEGFP-SA-neo carrying a transcription unit for hairpin RNA of the EGFP gene. One hundred fifty transformants were picked out from each transformation, and their GFP fluorescence was measured with the imaging system described under “Experimental Procedures.” The transformants were then classified into five categories (0–20, 21–40, 41–60, 61–80, and above 81%) based on fluorescence relative to the corresponding parent recipient strain. The number of double transformants in each category was plotted in the graph. The asterisk indicates a *mdl-2* transformant with silenced phenotype that is further analyzed by Southern blots in B. B, Southern blot analysis of the *M. oryzae* transformants with silenced or nonsilenced phenotype under different genetic backgrounds. Genomic DNA was digested with EcoRI and XbaI, fractionated on a 0.7% TAE-agarose gel, and probed with the EGFP gene. The open triangle indicates the 2.6-kb fragment corresponding to the transcriptional unit of EGFP mRNA, and the closed triangle represents the 3.2-kb fragment in pEGFP-SA-neo containing the transcriptional unit for hairpin RNA of the EGFP gene. S, silenced transformants; N, nonsilenced transformants.
Dicer-1 and Dicer-2 of Drosophila. They demonstrated that Dicer-1 played an essential role in processing miRNAs, a family of noncoding RNAs usually involved in developmental processes, whereas Dicer-2 was mostly responsible for siRNA production required for the RNA silencing pathway triggered by hairpin RNA. In contrast, Catalanotto et al. (16) showed that two DCL proteins (DCL1 and DCL2) in the filamentous fungi N. crassa were redundantly involved in the RNA silencing (quelling) pathway, since neither of the single DCL mutants but only the double DCL mutants were defective in gene silencing induced by either tandem-repeated trans-genes or a hairpin RNA-expressing construct. Therefore, our results are more consistent with the Drosophila dicers model than with the N. crassa model. This surprising result suggests complex diversification of DCL proteins during evolution, since M. oryzae is taxonomically much more closely related to N. crassa than to Drosophila.

Functional Diversification of DCL Proteins Responsible for siRNA Production in the RNA Silencing Pathway—In M. oryzae, no detectable siRNA was observed in mdl-2 mutants, whereas siRNA accumulation in mdl-1 mutants was at a level similar to that in wild type. The results obtained appear more complex. Dicer-2 mutants in Drosophila exhibited a large reduction in hairpin RNA-derived siRNA accumulation in vivo but not a complete loss. Similarly, the activity of siRNA production in protein extracts from the N. crassa dcl-2 mutant was significantly lower than wild type but was not completely impaired. These results suggested that siRNAs in the hairpin RNA-induced silencing pathway were generated mainly by

**Fig. 4.** MDL2 but not MDL1 is responsible for siRNA biogenesis and mRNA degradation induced by hairpin RNA. Northern blot analysis of siRNAs (A), mRNA (B), and dsRNA (C) of the EGFP gene in M. oryzae transformants with different genetic backgrounds in response to silencing induction. The blot in A was overexposed for 3 weeks to show no detectable siRNA accumulation in the mdl-2 mutants. DNA oligonucleotides (30-, 24-, and 20-mer) were used as molecular size makers for siRNA analysis. Equal loading of total RNA was estimated by ethidium bromide staining of rRNAs, predominant RNAs, or cleaved RNAs by S1 nuclease upon their availability. WT, wild type.

**Fig. 5.** Complementation of the mdl-2 mutant with the wild-type MDL-2 allele restored silencing phenotype and related siRNA accumulation in the mutant. A, silencing ability of a mdl-2 mutant and its complemented strain with the wild-type MDL-2 allele was assessed as described in the legend to Fig. 3A. Thirty-two colonies each were picked out after transformation with the silencing vector and subjected to the assay. B, Northern analysis of EGFP siRNA accumulation in the transformants with the silencing vector under different genetic background. DNA oligonucleotides were used as molecular size markers. Equal loading of total RNA was estimated by ethidium bromide staining of predominant RNAs.
Dicer-like Proteins in M. oryzae

In plants, two distinct siRNAs are involved in RNA silencing with different roles (34). A smaller 21-nt siRNA may be involved in RISC-mediated target cleavage, whereas a larger 25-nt siRNA positively correlates with DNA methylation and transmission of silencing signals systemically. Similarly, in the filamentous fungus *Mucor circinelloides*, two classes (21 and 25 nt long) of small antisense RNAs derived from the carotenogenic gene *carB* on self-replicative plasmids were detected in association with its silenced phenotype (35). Interestingly, two sizes of small antisense RNAs were differentially accumulated during vegetative growth. Long antisense RNA was undetectable in the spore, whereas shorter antisense RNA was predominant. We also previously reported that two or three different sizes of siRNAs were observed in *M. oryzae* (22). Therefore, as suggested in plants, it is likely that different sizes of siRNAs are also produced by distinct dicer activities in filamentous fungi. However, this does not seem to be the case with *M. oryzae*, because all the different sizes of siRNAs were observed in *mdl-1* mutants (data not shown), and none were detected in *mdl-2* mutants, indicating that all of the siRNAs were generated by MDL2 activity.

In *Drosophila*, Dicer-1 and MDL1 are possible candidates for the second dicer activity, but involvement of some other elements such as other RNase III-like enzymes or RdRP, as suggested by Makeyev and Bamford (27), cannot be excluded.

In the plant *Arabidopsis*, the DCL protein responsible for siRNA generation appears to depend on the trigger molecules of the silencing pathway. Mutants of *Arabidopsis* DCL3 showed a significant reduction in siRNA biogenesis for endogenous transposons but not in virus-derived siRNA formation. Conversely, in *Arabidopsis* dci-2 mutants, defects in siRNA generation activity were observed against infecting turnip crinkle virus but not against endogenous transposons (19). Therefore, DCL2 and DCL3 are separately responsible for virus-triggered siRNA and endogenous siRNA formation, respectively. In addition, using wheat germ extract, Tang et al. (4) suggested that two different classes of siRNAs found in plants might be generated by distinct dicer activities. Thus, complex pathways for the formation of various siRNA species appear to exist in plants.

In contrast, in *M. oryzae*, the presence of intact MDL2 correlated with siRNA accumulation and also with the occurrence of RNA silencing triggered by hairpin RNA. In addition, we observed that MDL2 was also required for endogenous siRNA biogenesis in *M. oryzae* (data not shown), indicating that MDL2 was responsible for the formation of most, if not all, of the siRNAs in *M. oryzae*. These results provide a clear model for the role of MDL2 in the RNA silencing pathway and contrast with some complexity in functional diversification of DCL proteins reported in other organisms. The RNA silencing pathway in *M. oryzae* may be mediated by simpler factors than in other organisms. Clear-cut results on the role of MDL2 obtained in the present study indicate that *M. oryzae* offers a simple and excellent system for elucidating the RNA silencing mechanism with rapid growth, haploid genome, genetic tractability, and known makers of the silencing mechanism.

The *Role of MDL1 in the siRNA/miRNA Pathways in M. oryzae*—In animals and plants, Dicer and Dicer-like protein are shown to be involved in the regulation of development by generating miRNAs. In the well studied case of *Drosophila*, miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNas. In the present study indicate that *M. oryzae* offers a simple and excellent system for elucidating the RNA silencing mechanism with rapid growth, haploid genome, genetic tractability, and known makers of the silencing mechanism. The *Role of MDL1 in the siRNA/miRNA Pathways in M. oryzae*—In animals and plants, Dicer and Dicer-like protein are shown to be involved in the regulation of development by generating miRNAs. In the well studied case of *Drosophila*, miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNAs, miRNAs are originally transcribed as long primary transcripts (pre-miRNAs) that are processed to the mature miRNas. In the present study indicate that *M. oryzae* offers a simple and excellent system for elucidating the RNA silencing mechanism with rapid growth, haploid genome, genetic tractability, and known makers of the silencing mechanism.

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One of the Two Dicer-like Proteins in the Filamentous Fungi *Magnaporthe oryzae* Genome Is Responsible for Hairpin RNA-triggered RNA Silencing and Related Small Interfering RNA Accumulation

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