Mutants defective in a *Mucor circinelloides dicer*-like gene are not compromised in siRNA silencing but display developmental defects

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Abstract

Dicer proteins are ribonuclease III enzymes that process double stranded RNA precursors into small RNAs categorized as small interfering RNAs (siRNAs) or microRNAs (miRNAs), which suppress gene expression through the RNA silencing mechanism. We have isolated a dicer-like gene (*dcl-1*) of *Mucor circinelloides*, the first gene of this family to be identified in zygomycetes. The *dcl-1* mRNA occurred in multiple forms, including the truncated molecules that result from premature polyadenylation. Null *dcl-1* mutants were not impaired as regards transgene-induced gene silencing, since they exhibited the same silencing frequency as the wild-type strain and accumulated the two size classes of siRNA associated with RNA silencing in *M. circinelloides*. However, *dcl-1* mutants showed a reduced growth rate and a hyphal growth alteration, which suggests that the *dcl-1* gene has some role in the control of endogenous functions.

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1. Introduction

RNA silencing is a complex regulatory mechanism that results in the suppression of gene expression through the sequence-specific degradation of the target mRNA, the repression of its translation or the inhibition of its transcription. Initially described as a molecular defense mechanism against virus and transposons, in recent years it has been realized that this mechanism has diverse biological functions, including the regulation of endogenous gene expression and heterochromatin formation (reviews in Nakayashiki, 2005; Filipowicz et al., 2005; Tomari and Zamore, 2005; Brodersen and Voinnet, 2006).

The genetic dissection of silencing, as well as biochemical analysis, supports the existence of a common mechanism with homologous genes and proteins acting in different organisms. The gene silencing mechanism is triggered by the deliberate or fortuitous production of double-stranded RNA molecules (dsRNA), which are processed into small molecules of 21 to 26 nucleotides with 2-nt 3’ overhangs (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Hutvagner et al., 2001). In the post-transcriptional RNA silencing pathways, these small RNAs can be classified into two types, according to their function: the small interfering RNAs (siRNAs), which act in the pathway that prevents the expression of exogenous nucleic acids (viral genomes, transposons and transgenes) and the micro RNAs (miRNAs), which act on endogenous regulation pathways. Both gene silencing pathways share a common protein set dedicated to producing and amplifying these small RNAs, which is called the “RNAi (RNA interference) machinery”. Within this machinery, the Dicer enzyme, a multidomain protein that processes dsRNA molecules into the small size RNAs (siRNAs and miRNAs), is of particular note (Berstein et al., 2001). Dicer belongs to an RNase III family with distinctive ATP-dependent RNA helicase, PAZ (Piwi/Argonaute/Zwille), dsRNA-binding and RNase III domains. The small RNAs generated by Dicer are incorporated into the RISC complexes.
(RNA-induced silencing complex), in which a protein of the Argonaute family (Ago protein) participates (Hammond et al., 2000). The RISC complex uses the antisense strand of the small RNAs as a guide to identifying the complementary sequences in the mRNA. The siRNAs target perfectly complementary mRNAs for degradation through endonucleolytic and exonucleolytic cleavages (siRNAs pathway). The mature miRNAs anneal normally to the 3′-untranslated region of target transcripts, preventing their translation or, especially in plants, inducing sequence-specific nucleolyis (miRNAs pathway).

The dsRNA precursors, the activators of the RNA silencing mechanism, may have various origins. Exogenous DNA or RNA molecules, such as transgenes, transposons and viruses, may generate dsRNAs as intermediaries in their replication mechanisms, through bi-directional transcription or as a result of the transcription of inverted repeated sequences integrated into the genome. In addition, sense transgenes integrated as single copy are also able to induce silencing by producing aberrant single-stranded transcripts that are converted in dsRNA by the activity of the RNA-dependent RNA polymerase (RdRP) enzyme. In the miRNA silencing pathway, the activator dsRNA molecules are transcribed by RNA polymerase II from endogenous non-protein-coding genes as imperfect stem–loop precursors, which are processed by the Dicer enzyme (Brodersen and Voinnet, 2006).

Even though the RNAi machinery is well conserved throughout the evolutionary scale, there are important differences that relate to the specialized functional activities of those proteins. For example, Schizosaccharomyces pombe, Caenorhabditis elegans and mammals have only one Dicer protein, which is able to process both miRNA precursors and the long dsRNA molecules derived from transgenes and other sources. In Drosophila melanogaster, there are two Dicer proteins; DCR-1 is mainly involved in the miRNAs pathway, whereas DCR-2 processes dsRNA precursors into siRNAs molecules (Lee et al., 2004). On the other hand, in Arabidopsis thaliana four Dicer proteins have been identified, one of them involved in miRNA production and the other three in the biogenesis of siRNA from different origins (Xie et al., 2004). Also in filamentous fungi, such as Neurospora crassa and Magnaporthe oryzae, there are two dicer genes, although the existence of miRNA in these organisms has not yet been described. In N. crassa, both dicer genes, named del-1 and del-2, are functionally redundant in siRNA production from transgene-derived dsRNA (Catalanotto et al., 2004), although DCL-2 appeared to be more able to produce siRNAs in vitro. In M. oryzae, a fungus closely related to N. crassa, only one of the two dicer genes, mtl-2, is required for siRNA biogenesis (Kadotani et al., 2004).

We have demonstrated the existence of an RNA silencing mechanism in the zygomycete fungus Mucor circinelloides (Nicolás et al., 2003; Ruiz-Vázquez, 2005). One of the remarkable feature of gene silencing in M. circinelloides is its induction by non-integrative transgenes. The introduction into the wild-type strain of self-replicative plasmids containing either wild-type or truncated copies of the carotenogenic carB gene, which encodes the phytoene dehydrogenase enzyme, causes silencing of the carB function. The albino phenotype shown by the silenced transformants, which is due to the accumulation of the colourless precursor phytoene, strongly contrasts with the bright yellow colour of the wild-type strain, which accumulates β-carotene in a light-dependent manner (Navarro et al., 1995). The silenced transformants accumulate small sense and antisense RNA molecules (siRNAs) corresponding to sequences of the carB gene. Of interest is the fact that gene silencing in M. circinelloides is associated with two size classes of antisense siRNA, 21- and 25-nt long, which are differentially accumulated during the vegetative growth of the silenced transformants. Both primary siRNA corresponding to the initial triggering molecule and secondary siRNA targeting regions downstream of the input trigger can be 21- and 25-nt in size and both size classes are preferentially produced from the 3′-end of the endogenous gene (Nicolás et al., 2003).

The two size classes of differentially accumulated siRNA detected in M. circinelloides could be produced by two distinct Dicer enzymes, showing different expression patterns and/or acting in different cellular compartments. By using degenerate oligonucleotides, we have cloned a dicer-like gene of M. circinelloides (del-1). Expression of this gene does not require activation of the silencing mechanism and produces several transcripts. In order to investigate the role of DCL-1 in RNA silencing, we disrupted this gene by homologous recombination. Phenotypic analysis of del-1 null mutants indicates that this gene is not essential for the biogenesis of transgene-derived siRNAs, which denotes the existence of an additional dicer gene in the M. circinelloides genome. Interestingly, the M. circinelloides del-1− mutant phenotype suggests a role for this gene in the control of growth and differentiation within the fungal mycelium.

2. Materials and methods

2.1. Strains, growth and transformation conditions

The leucine auxotroph R7B (Roncero, 1984), derived from M. circinelloides f. lusitanicus CBS 277.49 (syn. Mucor raceeosus ATCC 1216b) (Schipper, 1976), was used as the wild-type strain. Cultures were grown in minimal medium YNB (Lasker and Borgia, 1980), complete medium YPG (Bartnicki-Garcia and Nickerson, 1962) or in MMC medium (1% casamino acids, 0.05% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose), the last one supplementing the leucine auxotrophy of the leuA− pyrG− strains but not the uracil auxotrophy. Media were supplemented with l-leucine (20 μg/ml) or uridine (200 μg/ml), when required. The pH was adjusted to 4.5 and 3.2 for mycelial and colonial growth, respectively. Transformation was carried out as described previously (Nicolás et al., 2003). Illumination conditions were as previously
described (Quiles-Rosillo et al., 2003). *Escherichia coli* strain DH5α was used for all cloning experiments and strain LE392 (Promega, Madison, WI) for the propagation of *M. circinelloides* genomic lambda clones.

2.2. Vegetative growth analysis

The vegetative growth of the dcl-1 mutants was analyzed as previously described (Quiles-Rosillo et al., 2003). Plates for the strain MU402 (pyrG-) were supplemented with uridine (200 μg/ml). Vegetative growth was estimated by measuring the diameter of the colony using a ruler.

2.3. Plasmids

Plasmid pMAT660 contains a 2562 bp DNA fragment of the dcl-1 genomic sequence that extends from positions +1640 to +4201 relative to the translation initiation site of the dcl-1 gene. This fragment was amplified by PCR using the dicer4 and dicer5 oligonucleotides and cloned into the pGEM-T vector (Promega). Plasmid pMAT661 was used to disrupt the dcl-1 gene. This plasmid was constructed by cloning into the BamHI site located within the segment of the dcl-1 gene present in pMAT660, a 3.2 kb BamHI fragment isolated from plasmid pEM1 (kindly provided by A.P. Eslava, University of Salamanca, Spain), which includes the complete pyrG gene and promoter sequences. The resulting plasmid contains the selectable marker pyrG+ flanked by sufficient sequences of the dcl-1 gene (1.8 kb upstream and 0.76 kb downstream) to allow homologous recombination. The 5.8 kb disruption fragment was released from pMAT661 by SpeI and PvuII double digestion. Plasmids pMAT663 and pMAT665 contain, respectively, the 5.2 and 2.2 kb SacI genomic fragments isolated from the *M. circinelloides* lambda clone 13. Both plasmids were used to obtain the complete genomic sequence of the dcl-1 gene. Plasmid pMAT647 (Nicolás et al., 2003) includes the complete genomic sequence and promoter region of the carotenogenic gene carB. Plasmid pMAT652 (Nicolás et al., 2003) was used to prepare the carB antisense-specific riboprobe utilized to detect the carB siRNA.

2.4. Selection for pyrG- leuA- mutants

pyrG- leuA- mutants were isolated as described previously (Benito et al., 1995), with minor modifications. Briefly, spores of the leucine auxotroph R7B were treated with N-methyl-N'-nitro-N-nitroso-guanidine (0.1 mg/ml), inoculated on complete medium plates containing 200 μg/ml uridine and allowed to complete a vegetative cycle. The resulting spores were spread on minimal agar containing 2.5 mg/ml 5-fluorotic acid and 200 μg/ml uridine. Resistant colonies were tested for uracil auxotrophy, purified from single spores and assayed for OMPdecase activity. PyrG+ transformant of the pyrG- leuA- mutants were selected in MMC medium.

2.5. Nucleic acid manipulation and analysis

Genomic DNA from *M. circinelloides* was prepared as previously described (Ruiz-Pérez et al., 1995). Three degenerate oligonucleotide primers were designed to amplify the dicer-like gene of *M. circinelloides*. Sense primers dicer3 (5'-TAC(TGT(CT)CA(AG)TC(TCTGCG(CTAGACG-3')) and dicer4 (5'-GCTACTTCTGT(CT)(GC(T)GA(AG)GA(AG)GG-3') correspond to conserved sequences of the HELe domain (YVQSRGRA and ATSV(LV)EEG, respectively) and antisense primer dicer5 (5'-TCGAAGAT(AG)(G)C(AG)(TC(AG)CCGA(AG)(AG)AA(CT)(TC)-3') corresponds to the EFLGDAIF sequence of the RNaseIIb domain. Polymerase chain reaction (PCR) amplifications were carried out at 50°C using 2.5 U of EcoTaq Plus (Eco- gen, Spain). A LambdaGEM-11 genomic library of *M. circinelloides* wild-type strain CBS 277.49 (Quiles-Rosillo et al., 2003) was screened with two distinct probes to isolate recombinant plaques containing the complete dcl-1 gene. Probe f (Fig. 1A), which corresponds to the 2.5 kb fragment of the dcl-1 gene cloned in plasmid pMAT660, was used to screen 9000 plaques, allowing the identification of two positive clones that contained overlapping 5'-truncated versions of the *M. circinelloides* dcl-1 gene. Probe g (Fig. 1A), which corresponds to a 500 bp ApaI-BglII fragment isolated from plasmid pMAT660, was used for a new screen of 36,000 plaques to isolate the complete dcl-1 gene. Screening conditions were as previously described (Quiles-Rosillo et al., 2003). Standard recombinant DNA manipulations were performed as described by Sambrook and Russell (2001).

To identify transformants which correctly integrate the knockout vector designed to disrupt the dcl-1 gene, a rapid protocol was developed for isolating the DNA to be used in PCR amplifications. Fresh mycelium (1 cm2) from transformants grown in minimal medium was frozen in liquid nitrogen, ground to a fine powder in an Eppendorf tube and dissolved in 50 μl of DMSO. Aliquots (1.25 μl) of this preparation were directly used as DNA templates in PCR amplifications of 25 μl total volume.

Reverse transcription-PCR (RT-PCR) amplifications were performed to identify introns in the dicer genomic sequence. Total RNA was isolated from cultures grown for 7–14 h on minimal liquid medium (pH 4.5) and reverse transcribed with dcl-1-specific primers. To identify intron 1, primers dicer15 (5'-CCATGTATCTGCTTTC CAAGCG-3') and dicer20 (5'-ATGACCATCTCATCT CTTGACC-3') were used. Introns 2, 3 and 4 were identified with primers dicer14 (5'-GAATGTAAGCAATGA CCGTG-3') and dicer18 (5'-AACGTGGACGTTGG AAGGCA-3'). To identify intron 5, RNA was reverse transcribed with primer dicer13 (5'-AAACTCGTTGCTG CTGCCAT-3') and PCR-amplified with primers dicer13 and dicer7 (5'-GAAGAGGGCCCTGATATCAAG-3'). The 3' end of the full-length dcl-1 mRNA was identified by 3'-RACE experiments, using the dicer sense-specific primers dicer10D (5'-GTGTGCCTCTGTATCAACGC
CTG-3') and dicer12 (5'-ACCCCAACCCACCCCTACG CG-3') to amplify the cDNA reverse transcribed with the Race-T primer (Roche). Expand Reverse Transcriptase (Roche) and EcoTaq Plus (Ecogen, Spain) were used in all the experiments. The amplified fragments were cloned in pGEM-T or pGEM-T Easy vectors (Promega) and sequenced.

To characterize the dcl-1 transcripts in the dcl-1 mutants, RT-PCR reactions were carried out using total RNA isolated from mycelia of the wild-type and dcl-1 mutant strains grown for 24 h in minimal liquid medium. RNA was reverse transcribed with primers dicer15, dicer14 and dicer10R (5'-CAGGGCGTTGAATGCAGGC TTACCGACAC-3') and PCR amplified using the same antisense primers and the forward primers dicer20, dicer18 and dicer24 (5'-GGTGGTCCATTTGGCTTGC-3'), respectively.

2.6. Sequence analysis

Computer analysis of the sequences was carried out using European Bioinformatic Institute (EBI) Server software (EMBL Outstation, Cambridge, UK), National Center for Biotechnology Information (NCBI) (National Library of Medicine, Bethesda, MD, USA), Baylor College of Medicine (BCM) Search Launcher (Houston, TX, USA) and ExPASy Molecular Biology Server (Swiss Institute of Bioinformatics). BLAST and BLAST2WU programs were used for the data base search. Multiple sequences of the helicase and RNase III domains of Dicer proteins were
aligned using the ClustalW program to help in the design of
degenerate oligonucleotides. Conserved domains of the *M.
circinelloides* dcl-1 protein were identified by using the
InterProScan (EMBL-EBI) and the Conserved Domain
Database (NCBI) programs.

2.7. Northern and Southern analysis

Total RNA was extracted from mycelia of wild-type and
silenced strains grown under illumination conditions
(blue light at 4 W/m²) using Trizol reagent, following the
recommendations of the supplier (Invitrogen). Poly(A)⁺
RNA was purified using the “mRNA Purification Kit”
(Amersham Pharmacia Biotech), following the instruction
of the supplier. For Northern analysis, 50 μg of total RNA
or 5 μg of poly(A)⁺-enriched RNA from each sample was
hybridized to radioactively labelled probes at 65°C in
0.9 M NaCl, 1% SDS, 0.1 g/ml dextran sulfate. Filters were
washed under stringent conditions as previously described
(Nicolás et al., 2003). Probes were labelled with [α-32P]dCTP
using Ready-to-Go DNA labelling beads
(Amersham Pharmacia Biotech), following the instructions
of the supplier. The dcl-1 probe h is a 1.8 kb fragment
amplified with primers dicer20 and dicer14 and corresponds
to the Helicase domain of the Dicer protein.
Probe i is a 786 bp fragment of the dcl-1 gene amplified
with primers dicer19 (5’-AGTTGCCGCTTTGATCA
CAGAGGG-3’) and dicer10D and corresponds to the
RNase IIIb and DSRM domains.

Low molecular weight RNA was prepared from total
RNA, separated by electrophoresis on a 15% polyacryl-
amide gel and hybridized to radioactively labelled ribo-
probes as described previously (Nicolás et al., 2003). Filters
were washed as described (Nicolás et al., 2003) except that
the treatment with the RNase A/RNase T1 cocktail was
performed in 20 mM Tris–HCl pH 7.5, 5 mM EDTA and
60 mM NaCl. The antisense-specific riboprobe was pre-
bred by *in vitro* transcription of linearized pMAT652 plas-
mid, using the MAXIscript transcription kit (Ambion)
following the recommendations of the suppliers. Ribop-
probes were treated as described (Nicolás et al., 2003) to be
broken to an average size of 50 nt. Oligonucleotides corre-
sponding to sense and antisense sequences of the *carB*
gene were used as size and polarity controls.

For Southern analysis, genomic DNA (5 μg) isolated
from the wild type strain and putative dcl-1 mutants was
digested with the appropriate restriction enzymes, fraction-
ated on a TAE-agarose gel, blotted onto a Hybond-N⁺
filter and hybridized to a radioactively labeled dcl-1 probe
at 65°C. Hybridization and washing conditions were as
described (Nicolás et al., 2003).

2.8. Nucleotide sequence accession number

The nucleotide sequence of the dcl-1 gene of *M. circi-
elloides* has been deposited in EMBL/GenBank/DDBJ
databases under the Accession No. AM269947.

3. Results

3.1. Cloning a *M. circinelloides* dicer-like gene

In order to clone the *M. circinelloides* dicer-homologous
gene, degenerate primers corresponding to conserved
regions of the known Dicer proteins were designed and
used in PCR experiments with *M. circinelloides* genomic
DNA as a template. To reduce the degree of degeneration,
sequences of the dicer genes of *S. pombe* and *N. crassa*
were considered as references. Amplification with primers dicer4
and dicer5 gave rise to a 2.5 kb fragment that was re-ampli-
fied with the inner primer dicer3. The 2.5 kb fragment was
sequenced after cloning into pGEM-T vector to give plasm-
ids pMAT660. Analysis of the deduced amino acid sequence revealed a truncated ORF with a high degree of
similarity (45%) to the corresponding fragment of the *N.
crasa* dicer1 gene (E-value 1.57 e⁻⁵⁵). The *M. circinelloides*
gene was named dcl-1, for dicer-like gene 1. Two different
probes of the dcl-1 gene (probe f and probe g, Fig. 1; see Section 2) were used to screen, in duplicate, a genomic
LambdaGEM-11 library of the wild-type strain of *M. cir-
inelloides*. Extensive Southern blot analysis of DNA from
several hybridizing clones identified two *Sau*I fragments, 5.2
and 2.2 kb long, harboring sequences of the *M. cir-
inelloides* dcl-1 gene (Fig. 1A, clone 13). Those fragments were
cloned into the pBluescript II SK+ vector giving plasmids
pMAT663 and pMAT665, respectively. A nucleotide sequence of 7259 bp was obtained by sequencing the two
DNA strands of both plasmids. This sequence includes the
complete 4907 bp structural region of the dcl-1 gene. The
*M. circinelloides* dcl-1 gene is flanked by two highly con-
served sequences that correspond to the *purL* gene, which
codes for the FGAM synthase (phosphoribosylformylglyci-
namidine synthase), and the con7 gene, which codes for a
fungal transcriptional regulator involved in light responses
(Fig. 1A). The sequence upstream of the predicted ATG ini-
tiation codon of the *M. circinelloides* dcl-1 gene contains
general transcription signals (TATA and CAAT boxes) and
pyrimidine-rich tracks that are common to many fungal
promoters (Gurr et al., 1987). This latter region has been
involved in the correct initiation of transcription in some
*Aspergillus nidulans* promoters (Punt et al., 1990).

Analysis of the dicer sequence predicted the existence of
five possible introns (I1–I5, Fig. 1B), which was confirmed
by sequencing cDNA clones from RT-PCR products (see
Section 2). The RT-PCR experiment designed to locate the
exon–intron boundaries of intron I5 produced two distinct
cDNAs, which were cloned and sequenced (Fig. 1B). In one
of them, intron I4 but not I5 had been eliminated while in
the other one both introns had been processed, suggesting a
possible alternative splicing of the fifth intron. The same
results were obtained using different combinations of prim-
ers and/or RNA samples prepared from mycelia grown for
different period of times in liquid medium, which indicates
that this differential splicing event is not developmentally
regulated (data not shown).
The predicted *M. circinelloides* Dicer protein has a size of 1529 amino acid and an estimated molecular weight of 173 kDa. It contains all the structural domains normally found in known Dicer proteins and an identical domain organization: two helicase domains (DEXDc/DEAD/DEAH and HELc) at the N-terminus of the protein, one domain of unknown function (DUF283), a PAZ domain, two RNase III domains and a dsRNA-binding domain (DSRM) at the C-terminal end (Fig. 1C). The alignment of the *M. circinelloides* DCL-1 protein with Dicer sequences from other organisms using the ClustalW program shows a percentage of similarity ranging from 35% (humans) to 38–52% (fungi) and a percentage of identity ranging from 21.8% (humans) to 23–36.6% (fungi). The highest similarity corresponds to a putative protein, 1594 amino acids long, found in the zygomycete *Rhizopus oryzae* (contig 3.16), whose genome sequence was released (Broad Institute, Cambridge, MA) after the *M. circinelloides* dicer-like gene was isolated. Despite the limited identity at DNA level between the dicer-like genes of both zygomycetes, they share common exon–intron boundaries (Fig. 1D), which indicates the close relationship existing between these two fungi. The *R. oryzae* genome encodes an additional putative protein, 826 amino acids long (contig 3.7), that shows high similarity (54%) with the C-terminal region of the *M. circinelloides* DCL-1 protein. The sequence of the predicted *R. oryzae* protein corresponds to a truncated version of a dicer-like gene, from which the 5′ region encoding the helicase domains is missing (Fig. 1D). Interestingly, this truncated version of a dicer-like gene is 5′-flanked by the purL-homologous gene, as occurs in the *M. circinelloides* dcl-1 gene.

### 3.2. Expression analysis of the *M. circinelloides* dcl-1 gene

One of the most relevant features of the *M. circinelloides* gene silencing mechanism is the differential accumulation of the two size classes of siRNAs during vegetative growth. To analyze the expression of the *dcl-1* gene during the growth cycle, transcript abundance of the corresponding mRNA was determined by Northern blot experiments. Total RNA was isolated from wild-type and silenced strains grown for several periods of times in minimal liquid medium under light conditions. Hybridization with a *dcl-1* probe corresponding to the central region of the gene (probe *f*, Fig. 1A) identified two distinct transcripts, 5.2 and 3.2 kb long, which hybridized with the probe under stringent hybridization conditions (Fig. 2A). The 5.2 kb transcript, which accumulated to a lesser degree than the 3.2 kb transcript, corresponds to the size expected for the *dcl-1* mRNA and is present through the vegetative life cycle, as is the 3.2 kb transcript. Both transcripts accumulated at similar levels in wild-type and silenced strains, suggesting that an active silencing mechanism is not required for expression of the *dcl-1* gene. To determine the sequence of the *dcl-1* gene represented in the 3.2 kb transcripts, total RNA samples of the wild-type strain were hybridized with probes corresponding to particular regions of the gene. These experiments identified two additional transcripts derived from the *dcl-1* gene (Fig. 2B). A probe corresponding to the 5′ region of the gene (probe *h*, Fig. 1A) detected full-length *dcl-1* mRNA and, in addition, two shorter RNAs that migrated at positions corresponding to 1.1 and 1.3 kb, approximately. Probe *i*, which matches the 3′ end of the gene (Fig. 1A), detected the same 5.2 and 3.2 kb fragments identified previously using the *dcl-1* probe *f*. All the alternative transcripts were consistently detected in different RNA samples obtained from 7 to 48-h cultures of the wild-type strain (data not shown). The full-length and the shorter transcripts were detected in the poly(A)+-enriched fraction (Fig. 2B), indicating that all the *dcl-1* transcripts are polyadenylated.

Precise mapping of the 3′ end of the *dcl-1*-derived poly(A)+ RNAs was made by using 3′ RACE. Sense-specific primers *dicer*10D (outer) and *dicer*12 (inner) were used to localize the 3′ end of the full-length transcript. Cloning and sequencing of the resulting 319 bp fragment identified the 3′ end of the *dicer* gene 18 bp downstream of one of the consensus polyadenylation sequences (ATAAAA) found in this region, which is located 72 bp downstream of the putative stop codon (Figs. 2C and E). The 3.2 kb transcripts corresponding to the 3′ region of the *dcl-1* gene seem to represent a heterogeneous sized population (Fig. 2B). However, all of those transcripts probably use the polyadenylation site identified at the 3′ end of the gene, since several 3′ RACE experiments carried out using poly(A)+ RNA gave rise to homogeneous PCR products that identify the same 3′ end (data not shown). Forward primers *dicer*20 (outer) and *dicer*18 (inner) were used to identify the 3′ end of the *dcl-1* transcripts corresponding to the 5′ region of the gene. The 3′ RACE reactions yielded a PCR product of 550 bp (Fig. 2D). Sequencing of three independent clones from this product mapped an alternative polyadenylation site of the *dcl-1* gene 29 bp downstream of an AATAAA sequence located within the *dcl-1* coding region, 1080 bp downstream of the ATG initiation codon (Fig. 2E). A single amplification product was obtained in these 3′ RACE experiments, even though several primer combinations were used. This suggests that both 5′ transcripts use the same polyadenylation site, the size difference between them being due to an aberrant splicing or differences in the transcription initiation site.

### 3.3. Construction of a null *dcl-1* mutant

To determine the function of the *dcl-1* gene in the transgene-induced silencing mechanism, null mutants were generated by gene replacement. This strategy requires a strain with two distinct auxotrophic markers. An uracil auxotroph strain was derived from the leucine auxotroph R7B strain, giving the *leuA/ pyrG* strain named MU402. The *pyrG* mutation of MU402 was tested by the OMPdescarboxylase assay. Complementation of the two markers with plasmids carrying the *carB* gene revealed that strain
MU402 presents the same ability to induce gene silencing as the wild type strain R7B (data not shown). A knockout vector, named pMAT661, was designed to disrupt the \textit{dcl-1} gene. A 5.8 kb \textit{Puv} II–\textit{Spe} I fragment from this plasmid, which contains the \textit{pyrG} gene of \textit{M. circinelloides} flanked by 1.8 and 0.76 kb of the \textit{dcl-1} gene, was used to transform the MU402 strain. Integration of this fragment by homologous recombination into the \textit{dcl-1} locus should result in the disruption of the endogenous \textit{dcl-1} gene by the \textit{pyrG} gene (Fig. 3). This insertion should provoke termination of the \textit{dcl-1} wild-type sequence before the coding region for the \textit{W}rst RNase III domain (Fig. 1C). A total of 42 independent \textit{PyrG}+ transformants were obtained, which were analyzed after two cycles of vegetative growth for a stable \textit{PyrG}+ phenotype, as an indicator of exogenous DNA integration. PCR experiments designed to distinguish homologous from ectopic integration were carried out using fresh mycelia from 16 transformants showing 50–100% \textit{PyrG}+ spores (see Section 2). Four homokaryotic transformants showing 100% stable \textit{PyrG}+ spores amplified the expected 2 kb fragment and were finally selected for Southern analysis. DNA from those independent transformants was separately digested with \textit{Xho} I and \textit{Eco} RV enzymes and hybridized with a \textit{dcl-1} probe. The probe comprised the region of the \textit{dcl-1} gene used to direct recombination and recognizes both wild-type and \textit{dcl-1}¡ loci, but can discriminate between them (Fig. 3). All the transformants tested showed the expected 6.8 kb \textit{Xho} I and 2.7 and 3 kb \textit{Eco} RV fragments and the absence of the 3.6 kb \textit{Xho} I and 2.5 kb \textit{Eco} RV wild-type fragments, which indicated that the \textit{dcl-1} gene had been successfully disrupted (Fig. 3B). To confirm that the disrupted gene was unable to synthesize a...
Fig. 3. Disruption of the dcl-1 gene by homologous recombination. (A) Schematic representation of the dcl-1 locus in the wild-type strain (above) and dcl-1 disrupted mutants (below) obtained by homologous recombination with a linear DNA fragment. Grey box, genomic dcl-1 locus; white box, pyrG selectable marker. Restriction sites for XhoI (X) and EcoRV (E) are indicated, as well as the sizes of the predicted fragments. Primers used to identify homologous integration events are shown (pyrGZ and dicer19). Solid lines indicate position of the probe f used in Southern analysis. (B) Southern blot analysis of the wild-type strain R7B and four independent dcl-1 disruptants. Genomic DNA (5 μg) was separately digested with XhoI and EcoRV enzymes and hybridized with a 2.5 kb DNA fragment of the dcl-1 gene (probe f, Fig. 1A). This probe identifies different fragments in the dcl-1 wild-type and mutant loci. λ, Lambda HindIII size marker. (C) RT-PCR analysis of dcl-1 expression in the dcl-1 mutants. Total RNA isolated from the wild-type strain R7B and the dcl-1 mutant M1 was retro-transcribed using dcl-1-specific primers and amplified with primers corresponding to dcl-1 sequences located upstream of the pyrG insertion site (dicer20/dicer15 and dicer18/dicer14) or flanking the disruption point (dicer24/dicer10R). Total RNA without reverse transcription was used as negative control. Mw, GeneRuler DNA ladder mix (Fermentas).
functional Dicer protein, a series of RT-PCR experiments was carried out to characterize the del-1 transcripts in the disrupted mutants (Fig. 3C). As expected, specific primers located at the 5' region of the gene were able to amplify del-1 transcripts from the wild type and del-1 mutant strain. However, primer pairs flanking the pyrG insertion site amplified the wild type but not the del-1 mutant transcripts. These results confirm that the disrupted genes were unable to synthesize a complete Dicer protein containing the wild-type strain (transformant B7) or the silenced albino transformants obtained by introduction of plasmid pMAT647 into the del-1 mutant strains after being grown for 24 h in complete medium supplemented with uridine. The slower growth rate was observed in the four independent del-1 mutants (leuA<sup>+1</sup>) compared with the parental strain MU402 (leuA<sup>-1</sup> pyrG<sup>-1</sup>) (Fig. 5). The mycelial growth was quantified by periodically measuring the colony diameter of the null del-1 mutants and the wild-type strain for two days in complete medium supplemented with uridine. The slower growth of the del-1 mutants was evident from the beginning of growth, a differences that was accentuated with increasing growth time. Differences in the growth rate were even more evident when del-1 mutants were compared

3.4. del-1 mutants are not impaired in gene silencing

Plasmid pMAT647, which carries the light-induced carotenogenic carB gene as a silencing reporter and the leuA<sup>-1</sup> gene as a selectable marker, was used to transform the del-1 mutant strains in order to analyze the involvement of del-1 in transgene-induced gene silencing. Transformation of the wild-type strain with this self-replicative plasmid causes silencing of the carB function in 3–5% of transformants (Nicolás et al., 2003). Silencing of carB is easily detected by the albino phenotype of transformants when incubated in the light, because the carotenoid biosynthetic pathway is blocked. Transformation of two independent del-1 mutants with plasmid pMAT647 gave rise to a frequency of albino transformants similar to that of the wild-type strain (Table 1). These results indicate that del-1 mutants are silencing competent and suggest that del-1 gene is not essential in the silencing mechanism. The silenced phenotype was unstable in the del-1 mutants, as occurs in the wild-type strain, the frequency of the segregants with a wild-type phenotype after a cycle of vegetative growth being similar in both mutant and wild-type genetic backgrounds (data not shown).

Transgene-induced gene silencing in M. circinelloides is associated with two size classes of siRNAs, 21 and 25 nt long, which differentially accumulate during the vegetative growth of silenced transformants (Nicolás et al., 2003). To assess the involvement of del-1 gene in the production of those RNA molecules, the ability of del-1 mutants to accumulate the two classes of siRNAs was tested. Samples of low molecular weight RNAs were isolated from albino transformants of two independent del-1 strains after being grown for 24 h in liquid medium. RNA blot hybridization with a carB antisense-specific probe indicates that both del-1 mutants are able to accumulate the two size classes of antisense RNAs, as does the wild-type strain (Fig. 4). This results indicate that del-1 mutants are not impaired in the transgene-induced silencing mechanism and confirm that del-1 gene does not play an essential role in gene silencing.

3.5. del-1 mutants are impaired in vegetative growth and hyphal morphology

Dicer proteins participate both in the miRNA pathway that controls endogenous regulatory processes and in the siRNA pathway involved in transgene-induced silencing, among other processes. To investigate the possible role of del-1 in the endogenous regulation of cellular processes, the growth phenotype and hyphal morphology of the del-1 mutants were analyzed. A slight reduction in the growth rate was observed in the four independent del-1 mutants (leuA<sup>-1</sup>) compared with the parental strain MU402 (leuA<sup>-1</sup> pyrG<sup>-1</sup>) (Fig. 5). The mycelial growth was quantified by periodically measuring the colony diameter of the null del-1 mutants and the wild-type strain for two days in complete medium supplemented with uridine. The slower growth of the del-1 mutants was evident from the beginning of growth, a differences that was accentuated with increasing growth time. Differences in the growth rate were even more evident when del-1 mutants were compared

![Fig. 4. siRNAs production in the del-1 mutants. Northern blot analysis of low molecular weight RNAs (15 μg) isolated from the wild-type strain R7B and silenced albino transformants obtained by introduction of plasmid pMAT647 into the wild-type strain (transformant B7) or the del-1 mutants M1 and M2 (transformants TM1 and TM2). Cultures were grown for 24 h in liquid medium under continuous illumination conditions. Ten picomoles per lane of 29-mer DNA oligonucleotide in antisense orientation (AS) and 25-mer DNA oligonucleotide in sense orientation (S) were used as size marker and to control the hybridization specificity. RNA blot was hybridized with a hydrolyzed carB antisense-specific riboprobe, which correspond to a 1662 bp DNA fragment of the carB gene.](image)

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**Table 1**

Phenotype of transformants obtained after the introduction of the self-replicative plasmid pMAT647 into the del-1 mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transformants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total</th>
<th>Silencing frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R7B (del-1&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>26</td>
<td>489</td>
<td>515</td>
</tr>
<tr>
<td>M1 (del-1&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>93</td>
<td>1513</td>
<td>1606</td>
</tr>
<tr>
<td>M2 (del-1&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>20</td>
<td>636</td>
<td>656</td>
</tr>
</tbody>
</table>

<sup>a</sup> The color of the transformants was observed after 48 h in the dark, followed by 24 h under illumination with white light.

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**Fig. 4.** siRNAs production in the del-1 mutants. Northern blot analysis of low molecular weight RNAs (15 μg) isolated from the wild-type strain R7B and silenced albino transformants obtained by introduction of plasmid pMAT647 into the wild-type strain (transformant B7) or the del-1 mutants M1 and M2 (transformants TM1 and TM2). Cultures were grown for 24 h in liquid medium under continuous illumination conditions. Ten picomoles per lane of 29-mer DNA oligonucleotide in antisense orientation (AS) and 25-mer DNA oligonucleotide in sense orientation (S) were used as size marker and to control the hybridization specificity. RNA blot was hybridized with a hydrolyzed carB antisense-specific riboprobe, which correspond to a 1662 bp DNA fragment of the carB gene (Nicolás et al., 2003). Equal loading of the small RNA species was confirmed by EtBr staining of the predominant RNA species (sRNA) in the samples after the small RNAs were separated by agarose gel electrophoresis.
with the isogenic \textit{dcl-1} \textsuperscript{+} strain R7B (\textit{leuA} \textsuperscript{−}), which is the progenitor of MU402. The average and standard error for colony diameter measurements from ten colonies of the \textit{dcl-1} \textsuperscript{−} M1 mutant and the wild-type R7B strain were 5.52 ± 0.09 and 7.08 ± 0.09, respectively. Growth differences were statistically significant since there is less than 0.001 probability (\textit{T}-student test) that the average values for the growth of the \textit{dcl-1} \textsuperscript{−} mutant and wild-type strain are identical.

Null \textit{dcl-1} mutants do not present a wild-type hyphal morphology, as revealed by the observation of the leading edge of the fungal colony (Fig. 6). The youngest hyphae of the mycelium, which are in active exponential growth and have not yet reached the sporulation phase, are short and compact in the mutant strains, whereas in the wild-type they are long and disperse. All the independent \textit{dcl-1} \textsuperscript{−} mutants showed the same defective hyphal morphology (data not shown). Changes in the morphology of the leading hyphae in the \textit{dcl-1} \textsuperscript{−} mutants could indicate a role for this gene in the control of hyphal organization and differentiation within the fungal colony.

4. Discussion

Small RNAs act as sequence-specific guides in a variety of gene regulation processes from fungi to mammals. These small RNAs are generated from longer dsRNA precursors through the action of the RNase III enzyme Dicer. A search for the typical RNA silencing components in the public fungal genome data bases resulted in the identification of Dicer-like proteins in several fungi, mainly belonging to Ascomycota and Basidiomycota, but failed in a subset of them, as \textit{S. cerevisiae} (Nakayashiki, 2005). Most of those fungi show the standard two \textit{dicer} genes composition of \textit{N. crassa}, even though there are some exceptions, as observed in \textit{S. pombe} and \textit{Aspergillus nidulans}, which contain a single \textit{dicer} gene. The fact that relics of the RNA silencing genes, including truncated \textit{dicer} genes, were identified in several fungal genomes supports the idea that silencing genes were lost through genome reorganization during fungal evolution (Hammond and Keller, 2005; Nakayashiki, 2005). This seems to be the case with \textit{R. oryzae}, the only zygomycete whose genome sequence is known, which contains a single full-length \textit{dicer}-like sequence and a shorter truncated version.

We used a PCR-based approach to clone a \textit{dicer}-like (\textit{dcl-1}) gene of the zygomycete \textit{M. circinelloides}. The predicted gene product contains all the structural domains normally found in other proteins of the Dicer family, including a RNA helicase domain coupled with a DEXD-box ATP-binding site, a PAZ domain, which is also present in Argonate family proteins and selectively binds 3’ 2-nt overhang, two catalytic RNase III domains and a C-terminal dsRNA-binding domain, which probably contributes to the non-specific binding of substrates (Collins and Cheng, 2005). The similarity of the \textit{M. circinelloides} DCL-1 protein with the Dicer enzymes of the ascomycete \textit{N. crassa} and \textit{M. oryzae} ranges between 37 and 42%, the highest values being obtained with the DCL-1 and MDL-1 members of the Dicer family of each fungi. Nevertheless, the highest degree of similarity (52%) is shown with the \textit{R. oryzae} hypothetical protein, which contains all the conserved domains of Dicer enzymes (contig 3.16). Furthermore, the \textit{R. oryzae} genome encodes a hypothetical protein 826 amino acids long (contig 3.7) showing high similarity (54%) to the C-terminal region of \textit{M. circinelloides} DCL-1 (Fig. 1). This hypothetical protein lacks the ATP-RNA helicase domain that characterizes the class III of the RNase III family, but contains two RNase III domains and a dsRNA-binding motif, which define class II of this family (Hammond, 2005). The class II of RNase III is exemplified by the \textit{D. melanogaster} Drosha protein, a nuclear enzyme that processes the long precursors of pre-miRNAs. Interestingly, the \textit{R. oryzae} 826 amino acid protein is flanked...
upstream by the purL-homologous gene, the same sequence that is located in the 5' region of the M. circinelloides del-1 gene. There is no data concerning the existence of transgene-induced silencing or any other RNA-mediated silencing process in R. oryzae, and neither has transcription of the dicer-homologous sequences been confirmed. The roles of the complete and truncated Dicer sequences in this fungus must await further investigation. Even so, similarity and sequence organization data could suggest that the del-1 gene of M. circinelloides corresponds to the truncated dicer sequence of R. oryzae.

Expression analysis of the del-1 gene reveals that del-1 mRNA occurs in multiple forms, including truncated molecules. The full-length transcript, 5.2 kb long, is constitutively accumulated through vegetative growth and its expression does not depend on the triggering of gene silencing by transgenes. Both full-length and truncated forms are polyadenylated, as they can be purified by oligo dT columns. An alternative polyadenylation site has been experimentally found 1081 bp downstream of the putative start codon, which could explain the origin of the two transcripts corresponding to the 5' region of the gene. Size differences between these transcripts could be due to variations in the transcription initiation site or alternative processing in the 5' region. Recently, extensive variation in the 5'-UTR of human dicer mRNAs has been described (Irvin-Wilson and Chaudhuri, 2005; Singh et al., 2005). This diversity is due to alternative splicing of 5'-leader exons and has a profound effect on translational efficiency.

The M. circinelloides 3.2 kb transcripts corresponding to the 3' region of the gene probably use the same 3' polyadenylation site as the full-length transcripts. The 3.2 kb truncated variant could arise from aberrant pre-mRNA processing, as is the case in A. thaliana. Three different transcripts of the A. thaliana del-1 gene responsible of miRNA biogenesis can be detected in Northern assays (Xie et al., 2003). Besides the full-length form, two non-overlapping truncated versions that arise through aberrant or incomplete splicing around an intron located in the PAZ domain-coding region are accumulated. We also detected the aberrant splicing of intron 15 in the M. circinelloides del-1 mRNA (Fig. 1). However, the 3.2 kb size of the truncated mRNA corresponding to the 3' region of del-1 does not fit with premRNA cleavage at intron 15, but instead correlates with the position of intron 14, which disrupts the helicase domain. We detected no alternative splicing of intron 14, although we can not rule out the possibility that such a process occurs but was not revealed in our RT-PCR experiments. Information about distinct forms of dicer mRNA is very scarce. However, the results obtained suggest that alternative processing of the dicer gene could be a mechanism which has been preserved in several organisms such as the fungus M. circinelloides and the plant A. thaliana. Further studies would be required to test whether the relatively stable truncated transcripts of the dicer genes serve any biological function.

To investigate the role of the del-1 gene in the silencing mechanism of M. circinelloides, we generated a del-1 mutant by homologous recombination. Phenotypic analysis of the del-1 mutants revealed that the transgene-induced silencing mechanism had not been impaired, as the mutants showed identical silencing frequency to the wild-type strain. In addition, they were perfectly able to produce the two classes of siRNAs, 21 and 25-nt long. These results demonstrate that the del-1 gene is not essential for sense transgene-induced gene silencing. We have also demonstrated that the del-1 gene is not involved in dsRNA-induced gene silencing, since transformation of the del-1 mutants with an inverted repeat transgene gave rise to silenced transformants that accumulated the two size classes of siRNAs with the same pattern than the wild-type strain (our unpublished results). These results imply that, at least one additional dicer gene would have to exist in M. circinelloides to produce the siRNA molecules. In the ascomycete N. crassa, both dicer genes are redundantly involved in the siRNA pathway, since only a double mutant is impaired in transgene-induced gene silencing (Catalanotto et al., 2004). However, the DCL-2 protein seems to play the principal role in dsRNA processing, as suggested by the decreased Dicer activity of the del-2 single mutant. No miRNA-directed silencing pathway has so far been identified in fungi. However, N. crassa presents an RNA silencing-related phenomenon that abolishes the expression of genes that exist in one parental chromosome but not in its pairing partner (Shiu et al., 2001). The mechanism of silencing in this phenomenon, named MSUD (meiotic silencing by unpaired DNA), is supposed to be quite similar to the mechanism induced by repeated transgenes except that MSUD uses a distinct set of silencing protein components (Nakayashiki, 2005). Thus, specific RdRP, Dicer and Ago proteins are involved in MSUD, suggesting that two separate silencing pathways exist in N. crassa (Shiu et al., 2001; Lee et al., 2003). It has been proposed that the MSUD pathway in fungi and the miRNA pathway in higher eukaryotes may have evolved from a common ancient machinery, as both are involved in development (Nakayashiki, 2005). It is tempting to speculate that DCL-2 is the primary Dicer protein responsible for the siRNA pathway in N. crassa even though DCL-1, which is basically involved in MSUD, can compensate the loss of DCL-2. In M. oryzae, a fungus closely related to N. crassa, only one of the two dicer genes is required for RNA silencing (Kadotani et al., 2004). No detectable siRNA was observed in mdl-2 mutants, whereas the level of siRNA accumulation in mdl-1 mutants was similar to that observed in wild-type, indicating that MDL-2 is solely responsible for hairpin RNA-triggered RNA silencing. The role of MDL-1 is unknown, although the presence of malformed spores in mdl-1 mutants may imply the existence of a MDL-1-mediated miRNA pathway in M. oryzae (Kadotani et al., 2004). Molecular phylogenetic analysis of fungal Dicer-like proteins indicates that the MDL-1 and MDL-2 proteins are the ortholog of the N. crassa DCL-1 and DCL-2 proteins, respectively (Nakayashiki, 2005).

The M. circinelloides del-1 mutants are affected in their development, as exemplified by a significant reduction in...
the vegetative growth rate and the alteration in hyphal morphology observed in the leading edge of the fungal colony. The growth defect shown by the four independent dcl-1 mutants does not appear to be the consequence of a metabolic alteration, since growth analysis was carried out in complete agar medium. Rather, it seems to be related with differences in hyphal organization and differentiation within the fungal colony, as hyphal differentiation depends on the growth rate (Vinck et al., 2005). Leading hyphae growing from the advancing edges of colonies, known as exploring hyphae in A. nidulans, has been involved in the fungal colonization of substrates (Vinck et al., 2005). The exploring hyphae of the dcl-1 mutants show a different morphology with respect to the wild-type. Little is known about the molecular basis of intrahyphal genetic differentiation in filamentous fungi, although differential gene expression within a fungal colony has been recently reported (Tey et al., 2005; Vinck et al., 2005). These experiments indicate that fungi regulate some tip cell-associated processes by the spatial control of gene expression. It is tempting to speculate that M. circinelloides dcl-1 gene is involved in a developmental pathway that controls growth and hyphal differentiation.

The phenotypic characteristics of the dcl-1 mutants suggest that the M. circinelloides dcl-1 gene is implicated in development, as it is the case of the N. crassa dcl-1 and M. oryzae mdr-1 genes. Nevertheless, we cannot discard the possibility that M. circinelloides dcl-1 participates in the siRNA pathway, where it would have a redundant function with a presumed second dicer gene. The presence of more than two dicer-like genes in M. circinelloides would be unusual, considering that all but two the filamentous fungi whose genomes have been sequenced to date contain at most two dicer-homologous genes (http://www.broad.mit.edu/annotation/fungi; Nakayashiki et al., 2006). Consequently, the phenotype of the dcl-1 mutants could suggest that a single Dicer is responsible for the production of the two size classes of siRNAs in M. circinelloides. The size of the siRNAs generated by the Dicer enzymes is measured by the spacing of the terminal binding PAZ domain and the active site (Collins and Cheng, 2005). Thus, differences in siRNA sizes may arise from intrinsic structural characteristics of the Dicer enzymes. However, it is also possible that interaction of Dicer enzymes with specific dsRNA-binding proteins affects the structural conformation of the enzyme and provokes slight differences in the sizes of the siRNA molecules (Brodersen and Voinnet, 2006). The association of Dicer enzymes with specific dsRNA-binding proteins has been reported in plants and animals, where the complexes are required for the RISC loading of small RNA (Qi and Hannon, 2005).

Transgene-induced gene silencing in A. thaliana is also associated with two size classes of siRNA, 24 and 21-nt long (Hamilton et al., 2002). It has been proposed that 21-nt siRNAs serve as guides for the RISC complex to identify the target mRNAs, whereas the 24-nt siRNA class seems to exclusively mediate chromatin modifications. In vitro experiments have identified two distinct Dicer activities in plants, which are responsible for the production of each class of siRNAs (Tang et al., 2003). These results have been confirmed by in vivo experiments that point to DCL-3 as being the enzyme responsible for producing the heterochromatin-associated 24-nt siRNA, while DCL-4 is the preferred enzyme for production of 21-nt siRNAs from dsRNA (Xie et al., 2004; Dunoyer et al., 2005). Identification of the second dicer-like gene of M. circinelloides, analysis of its role in transgene-induced gene silencing and characterization of its enzymatic activity are needed to ascertain whether the two classes of siRNA are produced by the same Dicer activity and to clearly establish the role of these two classes of siRNAs in gene silencing.

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