DCL-1 colocalizes with other components of the MSUD machinery and is required for silencing

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Abstract

In Neurospora, a gene present in an abnormal number of copies is usually a red flag for mischief. One way to deal with these potential intruders is by destroying their transcripts. Widely known as RNA interference (RNAi), this mechanism depends on the “dicing” of a double-stranded RNA intermediate into small-interfering RNA, which in turn guide the degradation of mRNA from the target gene. Quelling is a vegetative silencing system in Neurospora that utilizes such a mechanism. Quelling depends on the redundant activity of two Dicer-like ribonucleases, DCL-1 and DCL-2. Here, we show that Meiotic Silencing by Unpaired DNA (MSUD), a mechanism that silences expression from unpaired DNA during meiosis, requires the dcl-1 (but not the dcl-2) gene for its function. This result suggests that MSUD operates in a similar manner to Quelling and other RNAi systems. DCL-1 colocalizes with SAD-1 (an RdRP), SAD-2, and SMS-2 (an Argonaute) in the perinuclear region.

1. Introduction

Neurospora crassa is a multicellular organism made up of a network of hyphae called a mycelium. Since the septum between two adjacent cells is normally incomplete, nuclei as well as other cellular contents can migrate freely along their shared cytoplasm through protoplasmic streaming. N. crassa is thus especially vulnerable to invasion by viruses or other selfish DNA elements. Accordingly, several mechanisms have evolved that silence the expression of repetitive sequences. For example, Quelling is a vegetative silencing system that destroys the transcripts made from hyperhaploid transgenes (Catalanotto et al., 2006). Repeat-induced point mutation (RIP), which operates before karyogamy, is a mechanism that targets duplicated sequences for mutation (Galagan and Selker, 2004). We recently discovered a third silencing mechanism termed Meiotic Silencing by Unpaired DNA (MSUD), which protects the genome during the sexual cycle (Shiu et al., 2001).

Quelling is related to an RNA-based silencing phenomenon often referred to as RNA interference (RNAi; Mello and Conte, 2004). In Quelling, the presence of multiple copies of a transgene presumably alerts the host defense mechanism, triggering the production of a single-stranded aberrant RNA (Catalanotto et al., 2006). QDE-1, an RNA-directed RNA polymerase (RdRP), converts the aberrant RNA into a double-stranded species (dsRNA).
The dsRNA molecules are processed by Dicers into small-interfering RNA (siRNA) of 21–25 nt, which subsequently guide the dge-2-encoded Argonaute nuclease (and its interacting protein, QIP) to degrade homologous mRNA (Catalanotto et al., 2002; Maiti et al., 2007). Since an RdRP (SAD-1) and an Argonaute protein (SMS-2) are also required for meiotic silencing (Shiu and Metzenberg, 2002; Lee et al., 2003), it is possible that this sexual silencing process operates in a similar fashion. For the Quelling mechanism, it is known that the QDE-1 RdRP can synthesize short RNA directly from single-stranded RNA, a subset of which has size similar to that of siRNA (Makeyev and Bamford, 2002). It is unclear whether the SAD-1 RdRP can also produce such small RNA species, and whether such an activity can bypass the requirement of Dicers, as once suggested for the Quelling mechanism. In this paper, we have asked if the two Dicer genes encoded in the Neurospora genome are involved in meiotic silencing.

2. Materials and methods

2.1. Strains and media

Neurospora strains used in this study are listed in Table 1. Auxotrophic strains used in this study were obtained through the Fungal Genetics Stock Center (FGSC; McCluskey, 2003). Descriptions of the del-lΔ and del-2Δ deletion alleles can be found in Catalanotto et al. (2004). The del-lΔ (frameshift) allele was created by a restriction cut at the unique AgeI site (positions 1210–1215; Fig. 1) followed by a Klenow fill-in. Descriptions of other loci used in this study can be found in Perkins et al. (2001). Culturing and crossing media were prepared as previously described (Vogel, 1964; Wester gaard and Mitchell, 1947). Crosses, genetic selection, and other routine manipulation were performed according to Davis and DeSerres (1970). Homokaryon isolation from heterokaryotypic transformants was performed using the protocol of Ebbole and Sachs (1990).

2.2. DNA manipulation and transformation

Cloning and other molecular biology procedures were as described by Sambrook and Russell (2001). The DNeasy Plant Mini Kit (Qiagen, Valencia, CA) was used for Neurospora DNA isolation. Custom oligonucleotides were obtained from Invitrogen (Carlsbad, CA). Polymerase chain reaction (PCR) was performed in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA), using either the FastStart High Fidelity PCR System or the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN). The pCRII-TOPO vector (Invitrogen, Carlsbad, CA) was used for the cloning of PCR-amplified fragments. Plasmid DNA was purified from bacterial culture with the HiSpeed Plasmid Midi Kit (Qiagen). DNA sequencing services were provided by the University of Missouri DNA core (Columbia, MO).

Primers involved in the construction of del-lΔ, del-l-gfp, and smm-2-rfp are listed in Table 2, with the resulting PCR products inserted into pBM61 (Margolin et al., 1997), pMFP272 (Freitag et al., 2004) and pMFP334 (Freitag and Selker, 2005), respectively. Fungal gene placement at the his-3 locus, using electroporation of washed conidia, was performed according to Margolin et al. (1997).

2.3. Reverse-transcriptase (RT)-PCR

Total RNA was extracted as previously described (Logemann et al., 1987; Shiu and Glass, 1999) and enriched for poly(A)+ using the Oligotex mRNA kit (Qiagen, Valencia, CA). For RT-PCR, poly(A)+-enriched RNA was used for cDNA synthesis using the first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). Primers used in amplification of the cDNA regions spanning the introns are listed in Table 2. The PCR products of the cDNA (without introns), as compared to that of the DNA, are as follows: 159 bp vs. 221 bp for del-l intron 1, 319 bp vs. 371 bp for del-l intron 2, 207 bp vs. 389 bp for del-2 introns 1–3, and 550 bp vs. 600 bp for del-2 intron 4. All intron identities were confirmed by DNA sequencing of RT-PCR products from both vegetative mRNA (from 40-27) and perithecial mRNA (from 40-27×81-02).

2.4. Cytological methods

For crosses involving the histone H1-gfp gene (hH1-gfp), strains containing hH1-gfp were used as the male parent, whenever possible, to limit the green fluorescence to ascogenous hyphae and asci. At least 10 perithecia were dissected at 1–2 day intervals in a drop of 10% glycerol between 3 and 8 days after crossing. The rosettes of asci were gently squashed under a cover glass. At least 1000 asci were exam-
The nucleotide and amino acid sequence of dcl-1 (NCU08270) of *N. crassa*. The two introns are italicized, whereas sequences matching the consensus for the TATA box and the transcriptional start site are underlined. The DNA sequence corresponds to nucleotides 225299–219300 of contig 7.53 of the Neurospora genome (http://www.broad.mit.edu).
ined for each cross for determining whether hH1-gfp was expressed or silenced in the developing asci and ascosporas. A Nikon Microphot FX fluorescence microscope fitted with an excitation/dichroic mirror/long pass filter set up was used, as described in Freitag et al. (2004). See Raju (1980) for a review on normal ascus development in N. crassa.

For localization studies involving GFP and RFP, perithecia were fixed in freshly prepared 4% paraformaldehyde, 90 mM PIPES pH 6.9, 10 mM EGTA, 5 mM MgSO₄ for 20 min at room temperature. They were rinsed briefly in PBS, and the perithecial contents were teased out into a drop of 90% glycerol, 10% 100 mM potassium phosphate, and 1,4-diazabicyclo[2,2,2]octane. Contents of 3–5 perithecia were dispersed after storage at 0°C, whereas those from a wild-type (WT) cross. The beaks of the perithecia are small and under-developed. Dissection of the mutant. We did not observe any obvious aberrant phenotypes in the dcl-1 Δ dcl-2 Δ mutants during vegetative growth. Sexual development in a cross homozygous for the dcl-1 Δ mutation also appears to be normal. However, a cross homozygous for the dcl-1 Δ mutation is completely barren. Although pigmented perithecia are produced in a dcl-1 Δ × dcl-1 Δ cross, they are slightly smaller than those from a wild-type (WT) cross. The beaks of the perithecia are small and under-developed. Dissection of the perithecia shows that they contain a very small ball of tissue, which is mostly made up of paraphysal cells. There are no recognizable ascogenous tissues, croziers, or asci within the perithecia. These data suggest that the development of perithecia is arrested at an early stage in a dcl-1 Δ × dcl-1 Δ cross.

3.2. Mutation in dcl-1 affects sexual development

Two Dicer-like genes, dcl-1 and dcl-2, are involved in Quelling in N. crassa (Catalanotto et al., 2004). The two genes are redundant for the Quelling function, and defects in silencing can only be observed in a dcl-1 Δ dcl-2 Δ double mutant. We did not observe any obvious aberrant phenotypes in the dcl-1 Δ, dcl-2 Δ, or dcl-1 Δ dcl-2 Δ mutants during vegetative growth. Sexual development in a cross homozygous for the dcl-1 Δ mutation also appears to be normal.

Table 2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dcl1-5207F</td>
<td>368 GAGAATATCAGGTGGAGCTTTTTGAACGC 395</td>
<td>cDNA spanning del-1 intron 1</td>
</tr>
<tr>
<td>Dcl1-5427R</td>
<td>588 CAATTCGCTTGATTGCAAAACTCGGTGTTGTTGGAA 564</td>
<td>cDNA spanning del-1 intron 1</td>
</tr>
<tr>
<td>Dcl1-107713F</td>
<td>107713 CTCTTGCCTCAACCAGTACC-ACTAGT</td>
<td>cDNA spanning del-1 intron 2</td>
</tr>
<tr>
<td>Dcl1-32636R</td>
<td>590 TTGTGATTGCAAAACTCGGTGTTGTTGGAA 564</td>
<td>cDNA spanning del-1 intron 2</td>
</tr>
<tr>
<td>Dcl1-34775F</td>
<td>2729 TAACCGTTCATGGTGGTTTGGAA 2751</td>
<td>cDNA spanning del-1 intron 3</td>
</tr>
<tr>
<td>Dcl1-9720R</td>
<td>4881 GTAGAAT-TTAATTAA</td>
<td>cDNA spanning del-1 intron 4</td>
</tr>
<tr>
<td>Dcl1-10100R</td>
<td>5261 TTCACGAGGCTTACAGGATTTCCATAC 5235</td>
<td>cDNA spanning del-1 intron 4</td>
</tr>
</tbody>
</table>

Genome Database (Fig. 1), dcl-2 was found to contain 4 (instead of the predicted 3) introns by our RT-PCR experiment (Fig. 2). Translation of the dcl-2 ORF yields a 1539-amino-acid (aa) polypeptide (instead of the predicted 1421-aa polypeptide).

The cDNA products of dcl-1 and dcl-2 mRNA (the identities of which were confirmed by sequencing) could be detected in mycelia and in two perithecial preparations (4 and 6 days after fertilization), suggesting that the two genes are expressed in both vegetative and sexual tissues (see Section 2.3).

3.3. dcl-1 Δ, dcl-2 Δ, and dcl-1 Δ dcl-2 Δ do not act as dominant suppressors of meiotic silencing

sad-1 and sad-2 each encode a component of the meiotic silencing machinery (Shiu et al., 2001, 2006). Deletion and heavily mutated strains of sad-1 and sad-2 act as dominant
Fig. 2. The nucleotide and amino acid sequence of dcl-2 (NCU06766) of N. crassa. The four introns are italicized, whereas sequences matching the consensus for the TATA box and the transcriptional start site are underlined. The DNA sequence corresponds to nucleotides 97103–103102 of contig 7.33 of the Neurospora genome (http://www.broad.mit.edu).
suppressors of meiotic silencing. In a cross, these Sad mutant alleles presumably prevent the wild-type sad-1 gene from proper pairing, thus silencing the silencer and creating a negative feedback loop for the meiotic silencing mechanism. To determine if such dominant suppression can be observed in heterozygous crosses of Dicer mutants, we examined the ability of Dicer deletion mutants to prevent the meiotic silencing of an unpaired copy of hH1-gfp inserted at the his-3 locus (his-3^+:hH1-gfp). When crossed to a his-3^+:hH1-gfp strain, dcl-1^D, dcl-2^D, and dcl-1^D dcl-2^D behave like wild-type (Fig. 3A), in that the unpaired hH1-gfp gene is silenced throughout meiosis and postmeiotic mitosis. These observations suggest that unlike Sad-1 and Sad-2 (Fig. 3B), dcl-1^D, dcl-2^D, and dcl-1^D dcl-2^D mutants do not act as dominant suppressors of meiotic silencing. Additionally, meiotic silencing of his-3^+:hH1-gfp appears to function normally in a cross heterozygous for dcl-1^D and homozygous for dcl-2^A (dcl-1^D dcl-2^A × his-3^+:hH1-gfp dcl-2^A; Fig. 3C).

3.4. A cross deficient in dcl-1 and dcl-2 gene products is defective in meiotic silencing

In the previous experiment, we showed that the Dicer deletion mutants do not act as dominant suppressors of meiotic silencing. These results suggest that either (1) the two Dicer genes are not involved in meiotic silencing, or (2) an unpaired copy of a dcl gene is not sufficient to silence its own expression. To determine the role of Dicers in meiotic silencing unequivocally, we need to assay the expression of an unpaired gene in a dcl-1^D dcl-2^A dcl-1^D dcl-2^A background. Because dcl-1^A is required for early sexual development (see Section 3.2), this cross is technically impossible.

![Fig. 3. Rosettes of ascis from various dcl-1 and dcl-2 crosses, showing that the dcl-1 function is important for the meiotic silencing of the unpaired hH1-gfp gene.](image-url)

A  B  C  D  E  F
To address this problem, we devised an experiment in which dcl-1 could be expressed at an early stage of the sexual cycle and inactivated at later stages by MSUD. Accordingly, we have constructed a cross homozygous for dcl-2Δ, heterozygous for dcl-1Δ, heterozygous for an insertion of dcl-1FS (a frameshift null allele), and heterozygous for an insertion of hH1-gfp at the his-3 locus, i.e. his-3::dcl-1FS dcl-2Δ × his-3::hH1-gfp dcl-1Δ dcl-2Δ. There are no dcl-2+ copies in this cross. In addition, the lone dcl-1+ copy is subject to meiotic silencing triggered by two unpairing events (dcl-1+ vs. dcl-1Δ::hph at the endogenous dcl-1 locus and dcl-1Δ vs. hH1-gfp at the his-3 locus). Our results show that the unpaired hH1-gfp gene is indeed expressed throughout meiosis in such a background, indicating that meiotic silencing is deficient in a low Dicer environment (Fig. 3D). This result suggests that Dicers play a role in meiotic silencing.

3.5. dcl-1 is involved in Meiotic Silencing

Our previous results establish the fact that Dicers are involved in the mechanism of meiotic silencing. To determine whether dcl-1 and dcl-2 provide redundant function for meiotic silencing, as is the case for their roles in Quelling, we tested the dcl-1Δ and dcl-2Δ mutations individually for their ability to suppress the silencing of unpaired hH1-gfp. In a cross deficient only in dcl-1 gene products, i.e. his-3::dcl-1Δ × his-3::hH1-gfp dcl-2Δ, we observed the expression of the unpaired hH1-gfp gene throughout meiosis (Fig. 3E). However, we did not observe any expression of hH1-gfp in a cross deficient only in dcl-2 gene products, i.e. dcl-2Δ × his-3::hH1-gfp dcl-1Δ (Fig. 3F). These results suggest that while dcl-1 is important for meiotic silencing, dcl-2 is not required for the process.

3.6. DCL-1 colocalizes with other MSUD proteins

SAD-1 and SAD-2, two components of the meiotic silencing machinery, colocalize in the perinuclear region (Shiu et al., 2006). Since DCL-1 and SMS-2 are also involved in meiotic silencing, we tested whether the two proteins also colocalize in the same region. We made various fusion constructs using vectors encoding green and red fluorescent proteins (GFP and RFP) and determined the localization of their gene products in several pairwise crosses. Our results demonstrate that DCL-1 and SMS-2 colocalize with SAD-1 and SAD-2 in the perinuclear region (Fig. 4).

4. Discussion

Two Dicer-like genes, dcl-1 and dcl-2, are found in Neurospora. The two Dicer-like genes are apparently redundant in function for Quelling, since the double knockout mutant (dcl-1Δ dcl-2Δ) is deficient in silencing while single knockout mutants (dcl-1Δ and dcl-2Δ) are not (Catalanotto et al., 2004). In this study, we demonstrate that the two genes are not redundant for the meiotic silencing function, with only dcl-1 required for the process.

Previously, we have shown that sad-1, which encodes an RNA-directed RNA polymerase (RdRP), is required for meiotic silencing (Shiu and Metzenberg, 2002). The requirement of an RdRP implies the involvement of a double-stranded RNA species in the pathway. Our current model suggests that such a dsRNA species is made from a single-stranded aberrant RNA, which in turn is transcribed from an unpaired DNA region during the silencing process. The requirement of an Argonaute protein for meiotic silencing is further indication that this mechanism is dependent on the siRNA-guided destruction of mRNA transcripts (Lee et al., 2003). Our results here demonstrate that the DCL-1 protein is likely the enzyme responsible for producing siRNA from the dsRNA species.

Sad-1Δ and Sad-2Δ act as dominant suppressors of meiotic silencing (Shiu et al., 2001, 2006). We reason that
in a Sad\(^{A} \times \text{sad}^{+}\) cross, the wild-type sad gene is unpaired during meiosis. Subsequently, the sad silencer would silence itself, thus preventing an abundant production of the sad gene product and suppressing the silencing mechanism. Unlike Sad-1 and Sad-2, the dcl-1\(^{A}\) mutant does not act as a dominant suppressor of meiotic silencing. One possible explanation is that, in a dcl-1\(^{A} \times \text{dcl}^{+}\) cross, the one unpairing event is insufficient in silencing the dcl-1\(^{+}\) expression. Because dcl-1 products are needed for early sexual development, we cannot create a dcl-1-null environment through a cross homozygous for the dcl-1 mutation. We addressed this problem by putting one functional unpaired dcl-1\(^{+}\) copy and a second, non-functional unpaired dcl-1\(^{fs}\) copy through a cross. The dcl-1\(^{+}\) copy provides enough gene product for early sexual development. However, because an unpaired, ectopically-inserted dcl-1\(^{fs}\) copy is also present in the cross, the lone dcl-1\(^{+}\) gene will be silenced by two unpaired copies (dcl-1\(^{+}\) and dcl-1\(^{fs}\)) at later stages, thus preventing meiotic silencing from functioning properly. We predict that this strategy can be used to knock down the function of any particular gene of interest specifically during the later stages of sexual development.

Our previous study demonstrates that SAD-1 and SAD-2 colocalize in the perinuclear region, where siRNAs are shown to localize in mammalian cells (Shiu et al., 2006). SAD-1 is localized in the cytosol in the absence of SAD-2, suggesting that SAD-2 may function to recruit SAD-1 to the perinuclear region. The importance of their localization is not clear. It is possible that, when single-stranded aberrant RNA are exported from the nucleus, the gauntlet of SAD-1 RdRP molecules can convert them into dsRNA before they have a chance to reach the exonucleases or the translational machinery in the bulk cytosol. Different Dicers are located in different compartments of the cell; some are localized in the nucleus, while others are localized in the cytoplasm (Papp et al., 2003; Billy et al., 2001). Our data demonstrate that DCL-1 colocalizes in the perinuclear region with all the known factors of meiotic silencing, including SAD-1 RdRP, SAD-2, and SMS-2 Argonaute (Sk-2 and Sk-3 have not been molecularly characterized; Raju et al., 2007). This result suggests that the perinuclear region is the center of RNAi activity for meiotic silencing. Interestingly, some components of the RNA-silencing machinery have been shown to localize in the perinuclear region of Drosophila and mouse germ cells (Lim and Kai, 2007; Pane et al., 2007; Kotaja and Sassone-Corsi, 2007). Colocalization of RNAi proteins may allow the coupling of various reactions, thereby increasing the efficiency of gene silencing.

Like sad-1 and sad-2, a cross homozygous for dcl-1\(^{A}\) is barren. The progression of meiosis is blocked at the same stage for sad-1 and sad-2, namely at early prophase (Shiu et al., 2001, 2006). This similarity may be attributed to the fact that the SAD-1 and SAD-2 proteins work intimately together and that SAD-2 controls the perinuclear localization of SAD-1. Although ascii are made in a Sad\(^{A} \times \text{sad}^{+}\) Sad cross, the same cannot be said for a cross homozygous for dcl-1\(^{A}\). In such a cross, the sexual development is blocked at a much earlier stage, and the perithecia have no observable sexual tissue. These results suggest that the barrenness of a dcl-1\(^{A} \times \text{dcl}^{+}\) cross could be due to the defects of a different cellular pathway. One possibility is that DCL-1 is important for processing endogenous hairpin dsRNA into microRNA required for sexual development. In plants and animals, a microRNA species can bind to its complementary mRNA, leading to their degradation or translational blockage (Bartel, 2004). Since microRNA are yet to be discovered in fungi, it would be interesting to determine if such non-coding RNA exist in Neurospora and whether they require DCL-1 for maturation.

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References


