Knockdown of LIM15/DMC1 in the mushroom Coprinus cinereus by double-stranded RNA-mediated gene silencing

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The basidiomycete Coprinus cinereus has many advantages as a model organism for studying sexual development and meiosis, but it has been difficult to investigate using reverse-genetics methods, such as gene disruption by homologous recombination. Here, gene repression by dsRNA-mediated gene silencing was tried as an alternative method for reverse-genetics studies. It was shown that transformation of the LIM15/DMC1 dsRNA expression construct (LIM15dsRNA) resulted in genomic insertion of LIM15dsRNA and paucity of the LIM15/DMC1 transcript. First, LIM15dsRNA was transformed into the homothallic strain AmutBmut to generate a homozygote in which both nuclei had a copy of LIM15dsRNA. The LIM15/DMC1-repressed strain showed abnormal homologous chromosome synopsis during meiosis. Basidiospore production was reduced to 16% by the induction of dsRNA. However, approximately 60% of basidiospores were viable. Next, a heterozygote was generated in which one nucleus had a copy of LIM15dsRNA. The phenotype was similar to that of the homozygote. These results are not only the first demonstration of dsRNA-mediated gene silencing in a member of the homobasidiomycete fungi, to which 90% of mushroom species belong, but also the first successful use of a reverse-genetics approach in C. cinereus research.

INTRODUCTION

The basidiomycete Coprinus cinereus has been used as a model organism for the study of sexual development and meiosis, because of the rapid morphogenesis of its multicellular structure and its synchronous meiosis by photoinduction (reviewed by Casselton & Zolan, 2002; Kamada, 2002; Kues, 2000). We have conducted a number of studies of 3R (DNA replication, repair, recombination) enzymes in C. cinereus meiosis. We have found that transcripts of 3R-related proteins, such as DNA polymerase α, proliferating cell nuclear antigen (PCNA), DNA ligase I, Flap endonuclease 1, DNA ligase IV and Lim15/Dmc1, are abundant at meiotic prophase I, and we have discussed the roles of 3R enzymes during meiosis (Hamada et al., 2002; Namekawa et al., 2003a, b, c; Nara et al., 1999; Yamaguchi et al., 2004). These studies were made possible by the fact that large populations of synchronous meiotic cells can be obtained from C. cinereus to do biochemical analysis. As an extension of our research, we wanted to repress expression of these enzymes during meiosis, and simultaneously to pursue biochemical and genetic analyses to determine the roles of 3R enzymes during meiosis.

C. cinereus has been analysed using forward genetics approaches because of its relatively short life cycle and its ease of mutagenesis by transformation of an asexual basidiospore of the haploid mycelium known as the oidium (reviewed by Casselton & Zolan, 2002; Kamada, 2002; Kues, 2000). Unfortunately, it has been difficult to repress specific genes through methods such as gene disruption by homologous recombination. Although a low frequency of homologous recombination does seem to occur in C. cinereus (Binninger et al., 1991), so far,
targeted gene disruption by homologous recombination in
*C. cinereus* has not been reported. To get around this
problem, we tried gene repression by dsRNA-mediated gene
silencing as an alternative reverse-genetics technique in *C.
cinereus*.

In RNA interference, also known as dsRNA-mediated
gene silencing, dsRNA is fragmented by the RNase III-
like nuclease Dicer into 21–25 nt interfering RNAs (siRNAs; Bernstein et al., 2001). These siRNAs are
incorporated into the RNA-induced silencing complex (RISC) to disassemble the target mRNA complementary to
the siRNAs in a sequence-specific manner (Hammond et al.,
2000). RNA interference is conserved in various organisms
(reviewed by Montgomery, 2004), including *Caenorhab-
ditis elegans* (Fire et al., 1998), plants (Waterhouse et al.,
1998), *Drosophila* (Misquitta & Paterson, 1999), mammals
(Elbashir et al., 2001), and also in fungi such as fission yeast
(Raponi & Arndt, 2003), *Dictyostelium* (Martens et al., 2002),
and the heterobasidiomycete *Cryptococcus* (Liu et al., 2002).
Because of this broad conservation, we predicted that
*C. cinereus* might have an RNAi-like mechanism. However,
RNAi has not been reported so far in homobasidiomycete
fungi.

In this study, we select *LIM15* as a target of dsRNA-mediated
gene silencing. In eukaryotes, there are two types of recA-
like recombinases: Rad51 and Lim15/Dmc1. While RAD51 is
expressed in both meiotic and somatic cells and functions
in the DNA-repair reaction, *LIM15*/DMC1 expression is
restricted to meiotic cells. However, both enzymes are
thought to be involved in meiotic recombination, and the
functional difference between these enzymes during meiosis
is still obscure (reviewed by Masson & West, 2001).

If meiosis is hampered in *C. cinereus*, the normal black-
coloured basidiospore is not produced, and the cap of the
mature fruiting body becomes white or grey. Therefore, if we
use the meiosis-specific transcript *LIM15* as a target of
dsRNA-mediated gene silencing, we can evaluate the effects of
*LIM15* dsRNA by judging the phenotype of the mush-
room cap colour without affecting growth and morpho-
genesis. Here we show that transformation of a *LIM15/
DMC1* dsRNA expression construct (*LIM15dsRNA*) into *C.
cinereus* resulted in paucity of *LIM15* transcripts and
abnormal homologous chromosome synopsis during meio-
sis. The applications of dsRNA-mediated gene silencing in
*C. cinereus* research are discussed.

**METHODS**

**Strains and culture of *C. cinereus*.** The *C. cinereus* strains *AmatBmut* (Swamy et al., 1984), 5337 (Murata et al., 1998) and *spo22-1* (Kanda et al., 1990) used in this study have been described previously. Malt yeast/glucose (MY) medium (Rao & Niederpruem, 1969) solidified with 2% (w/v) agar in 9 cm diameter Petri dishes was used for routine mycelial cultures. MY medium without agar in 9 cm Petri dishes was used for mycelial cultures to extract genomic DNA. These cultures were maintained at 28 °C under a 12 h light/12 h dark regime, unless otherwise stated. The culture method for fruiting-body formation used here has been described previously (Nara et al., 1999). The cultures were incubated from day 0 to day 7 at 37 °C in total darkness and from day 7 onwards at 25 °C under a 16 h light/8 h dark regime to allow photo-
induction of fruiting-body formation. A series of meiotic events occurs synchronously in all the fruiting bodies under the proper light cycles, as described previously (Namekawa et al., 2003b). Typical procedures of photo-induction of meiosis are as follows: karyogamy (K), which is defined as the time at which 5% of all basi-
dia have fused nuclei, began at 04:00 (K + 1), 1 h before the light
was turned on; photo-induction started at 05:00 (K + 1).

**Construction of **pCcLIM15dsRNA**.** The expression vector pCCEX (*C. cinereus* constitutive expression), which has the consti-
tutive *C. cinereus* β-tubulin promoter, the *C. cinereus* β-tubulin
terminator and a multi-cloning site in the intervening space between the promoter and terminator, was made as follows. A 393 bp frag-
ment of the β-tubulin promoter (Cummins et al., 1999) was cloned
to the pCRII vector (Invitrogen) at HindIII and EcoRI sites. Then,
the resulting plasmid was digested with EcoRI and NotI, and a
multi-cloning site containing EcoRI, SstII, XhoI, KpnI, Xbal and
NotI sites was ligated into the digested site. The 427 bp fragment of
the β-tubulin terminator sequence was cloned into the NotI and
Apal sites of the plasmid to generate pCCCEX.

The *LIM15* dsRNA expression construct in pCcCEX (pCcLIM15dsRNA) was made as follows. The antisense strand which corresponds to 750–1 bp in *LIM15* cDNA was cloned into the XhoI and KpnI sites of pCCCEX. Next, the sense strand which corresponds to 101–750 bp in *LIM15* cDNA was cloned into the XbaI and NotI sites of the resulting plasmid to generate pCcLIM15dsRNA.

**Transformations.** Transformations of *C. cinereus* strain *AmatBmut*
protoplasts were performed exactly as described elsewhere (Binninger et al., 1987). For the co-transformation of pCcLIM15dsRNA with pPAB1-2, we used total of 4 μg intact plasmids of pCcLIM15dsRNA and pPAB1-2 in a molar ratio of 2:1. As a control, the strain *AmatBmut* was co-transformed with pCCCEX and pPAB1-2, or transformed with pPAB1-2 alone. The transformed cells were spread onto minimal medium (Binninger et al., 1987). After incubation for 1 week at 28 °C, transformants were selected.

**Genetic technique for mating to make heterozygotes.** The inocula (1×1 mm) of strain 5337 (Murata et al., 1998) on MY agar plates were removed 2 days later, followed by the inoculation of white-cap transformants to the place from which 5337 had been removed. After two more days, mated dikaryons were isolated from the marginal region (Makino & Kamada, 2004).

**Basidiospore production and viability.** The number of basidio-
spores produced per milligram of cap tissue was determined using the procedure described by Ramesh & Zolan (1995). The viability of basidiospores was established using the spotted-drop method described in Ramesh & Zolan (1995), with minor modifications. To inoculate the MY medium, the basidiospores were suspended in PBS and spotted onto the medium, which had been directly solidified on the glass slide. After overnight incubation at 37 °C, the germinated basidiospores were counted with an Olympus BH2 microscope. For each strain tested, basidiospores from three caps were analysed; the mean and standard deviation are reported.

**Electron microscopy.** Spreads of *C. cinereus* chromosomes were prepared and stained with silver nitrate, as described elsewhere (Pulkila & Lu, 1985), and grids were viewed with a JEOL-1010 electron
microscope.

**Other methods.** Southern and Northern analyses were performed as described previously (Namekawa et al., 2003b; Nara et al., 1999). For the Northern analysis, we used the *C. cinereus* homologue of

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*References cited in the text are available in the original document.*
the glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) as a loading control. The blots were stripped and sequentially reprobed for LIM15, RAD51, and G3PDH, in that order. For the DAPI staining of meiotic cells, a small piece of meiotic tissue was picked up by forceps and squashed immediately with DAPI in PBS and viewed with an Olympus BH2 microscope.

RESULTS

The LIM15 dsRNA expression construct and repression of LIM15

To study whether gene repression by dsRNA occurs in *C. cinereus*, we transformed a LIM15 dsRNA expression construct into *C. cinereus*. First, as shown in Fig. 1(A), we made an expression vector which had the constitutive *C. cinereus* β-tubulin promoter, the *C. cinereus* β-tubulin terminator, and a multi-cloning site in the intervening space between the promoter and the terminator. Into this multi-cloning site, we ligated the antisense and sense strands of the LIM15 cDNA to express dsRNA from the inverted repeat (pCcLIM15dsRNA; Fig. 1B). This construct was designed to express dsRNA corresponding to 10–750 bp of the LIM15 cDNA, and a loop structure corresponding to 1–100 bp in the LIM15 cDNA, and was named the LIM15 dsRNA expression construct (LIM15dsRNA). Since we used the meiosis-specific transcript LIM15 as a target, we predicted meiosis-specific defects by the constitutive expression of LIM15dsRNA.

We transformed this construct into the homothallic (self-compatible) strain AmutBmut (Swamy et al., 1984). AmutBmut has a defect in mating type genes A and B, and produces haploid oidia (asexual spores) that germinate into monokaryotic hyphae, then spontaneously form homozygous dikaryons which eventually form fruiting bodies and undergo normal meiosis and basidiospore production. Because of this, even if a meiotic gene is mutated in this strain, it is still possible to make a mutant homozygote spontaneously. Therefore, AmutBmut has been used as a starting strain to screen for mutations affecting meiosis (Casselton & Zolan, 2002). In addition, the 4-aminobenzoic acid (PAB) synthetase gene is defective in AmutBmut, so the nutritional requirement for PAB can be used as a selection marker.

In *C. cinereus*, normal meiosis results in a black cap on the fruiting body (Fig. 2A). Meiotic defects cause the fruiting body to have a pale or white cap due to the absence of healthy black basidiospores. This feature makes it easy to detect meiotic defects by the cap colour of the fruiting body after mutagenesis. To investigate the effect of LIM15dsRNA, we co-transformed pCcLIM15dsRNA and pPAB1-2 (Granado et al., 1997), which contains the PAB synthetase gene, into the oidia. We obtained 10 white-cap transformants out of a total of 44 PAB⁺ transformants (Table 1). These white-cap transformants specifically correspond to the transformation of the pCcLIM15dsRNA. No white-cap mutants were obtained when we used pPAB1-2 alone or pPAB1-2 and pCcCEX (Table 1). Fig. 2(B) shows an example of a white-cap transformant (lineage no. 2; line #2). All 10 white-cap transformants appeared completely normal with regard to mitotic mycelial growth and fruiting-body formation. We fruited the transformants more than 30 times, then scored for cap colour. The cap colour from white-cap transformants was always white, and these phenotypes were stable during passaging and after silica-gel stocks.

It is known that transformation of *C. cinereus* with a circular plasmid results in random cutting of the plasmid itself and random integration of the plasmid DNA into the genome (Binninger et al., 1987). We confirmed the integration of LIM15dsRNA into the genome by Southern analysis (Fig. 3). We extracted genomic DNA from each white-cap

(A) pCcCEX-1

![Diagram](http://mic.sgmjournals.org)

(B) pCcLIM15dsRNA

![Diagram](http://mic.sgmjournals.org)

Fig. 1. Construction of pCcLIM15dsRNA. (A) pCcCEX-1 plasmid. The constitutive expression vector which has the constitutive *C. cinereus* β-tubulin promoter, a multi-cloning site, and the *C. cinereus* β-tubulin terminator was ligated into the multi-cloning site of vector pCRII (Invitrogen). (B) pCcLIM15dsRNA plasmid. The 750–1 bp and 101–750 bp sequences of LIM15 cDNA were ligated in inverted repeats to the multi-cloning site of the vector pCcCEX-1 plasmid. β-tub, β-tubulin.
transformant and digested it with either \textit{Hin}dIII, a site for which is located at the 5' end of the \textit{b}-tubulin promoter, or \textit{Apa}I, a site for which is located at the 3' end of the \textit{b}-tubulin terminator (Fig. 1), using either the \textit{b}-tubulin promoter probe or the \textit{b}-tubulin terminator probe. If the entire \textit{LIM15} dsRNA construct integrated into the genome, we would expect to detect identical bands using either the promoter or the terminator probe, in addition to the bands corresponding to the endogenous \textit{b}-tubulin locus. For example, line #2 displayed one such band (indicated by an asterisk), in addition to the endogenous \textit{b}-tubulin bands in Fig. 3, lanes 6–9. When \textit{Hin}dIII digestion of line #2 was probed with the promoter probe, there was an additional band (compare lanes 7 and 9, Fig. 3). These results indicate that line #2 contains at least one intact copy of \textit{LIM15} dsRNA and a non-intact construct containing only the promoter sequence.

In a similar way, we analysed all 10 white-cap transformants (Fig. 3A–D and data not shown). We estimated the insertion number of the construct in all white-cap mutants, and summarized this in Table 2. We could detect intact constructs in all 10 white-cap transformants. However, there tended to be multiple intact and non-intact constructs. Although the \textit{LIM15} dsRNA has the \textit{b}-tubulin promoter and terminator, the endogenous bands from the \textit{b}-tubulin were unchanged in all cases (Fig. 3A–D and data not shown). These results indicate that the \textit{b}-tubulin locus was not disturbed by homologous recombination in these white-cap transformants. Further Southern analysis indicated that the endogenous band of \textit{LIM15} was unchanged (Fig. 3E–F and Supplementary Fig. S1). This confirmed that transformation did not alter the endogenous \textit{LIM15} gene locus by homologous recombination.

To investigate whether endogenous \textit{LIM15} is repressed in white-cap transformants, we performed Northern analysis. Our previous results demonstrated that the expression of \textit{LIM15} culminates at 4 h after karyogamy (K + 4; Nara et al., 1999). Therefore, we extracted total RNA from meiotic tissue at K + 4. Because two transformants (lines #1 and #11) showed aberrant progression of meiosis around the karyogamy stage, we excluded them and performed Northern analysis on the other eight transformants (Fig. 4). All eight transformants exhibited a paucity of \textit{LIM15} transcript compared to \textit{AmutBmut} and the recessive meiotic mutant \textit{spo22-1} (Kanda et al., 1990). Similar results were obtained with the \textit{LIM15} 1–750 nt probe, which contains the sequence from our dsRNA, and the \textit{LIM15} 801–1041 nt probe, which does not. The same filter was hybridized with \textit{RAD51} cDNA, which has high sequence identity to \textit{LIM15} (Nara et al., 1999). A significant difference was not observed between the pattern of \textit{RAD51} and the

![Fig. 2. Effect of \textit{LIM15} dsRNA on the cap colour of the mature fruiting body. (A) \textit{AmutBmut} spontaneously formed homozygous dikaryons and fruiting bodies. Normal basidiospore formation resulted in a black cap. (B) Homozygote in which both nuclei had the \textit{LIM15} dsRNA showed abnormal basidiospore formation, resulting in a white cap. The strains were fruiting in the morning, then incubated for 24 h, during which sporulation occurred. The next day, the strains were scored for cap colour.](image)

### Table 1. Constructs used for \textit{LIM15} gene silencing and their effect on cap colour

Transformation was performed with pPAB1-2, pPAB1-2 + pCcCEX and pPAB1-2 + pCc\textit{LIM15} dsRNA. The cap colour was checked the day after sporulation.

<table>
<thead>
<tr>
<th>Plasmid (marker)</th>
<th>Plasmid (construct)</th>
<th>Number of clones tested</th>
<th>Number with black cap</th>
<th>Number with white cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPAB1-2</td>
<td>–</td>
<td>27</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>pPAB1-2</td>
<td>pCcCEX</td>
<td>48</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>pPAB1-2</td>
<td>pCc\textit{LIM15} dsRNA</td>
<td>44</td>
<td>34</td>
<td>10</td>
</tr>
</tbody>
</table>
constitutively expressed gene G3PDH. Taken together, these results indicate that the entire LIM15 coding sequence was specifically repressed by the transformation of LIM15dsRNA.

**trans Effect of LIM15dsRNA in heterozygotes**

For further analysis, we chose transformed line #2, because line #2 has an intact construct and the fewest number of non-intact constructs of the white-cap transformants (Table 2). In the heterozygote of recessive meiotic mutants, even if one nucleus has a defect in a meiotic gene, the other nucleus, which contains a wild-type copy of the gene, can rescue the meiotic phenotype. Therefore, in the past, we have had to create the homozygote of recessive meiotic mutants to analyse the phenotype. However, we assumed that a heterozygote in which one nucleus had a copy of LIM15dsRNA would show the meiotic phenotype, contrary to the heterozygote of recessive meiotic mutants, because dsRNA could work on the other nucleus in trans. To analyse the phenotype of a heterozygote of a transformant which has LIM15dsRNA, we mated wild-type monokaryotic strain 5337 (Murata et al., 1998), with line #2, and produced dikaryotic strain #2×5337. #2×5337 showed meiotic defects and a semi-grey fruiting body (Fig. 5B). As a control, we mated AmutBmut with 5337, and produced dikaryotic strain AmutBmut×5337. AmutBmut×5337 showed normal meiosis (Fig. 5A).

To compare the effect of LIM15dsRNA in these strains, we determined the basidiospore production and viability (Table 3). As shown in Table 3, basidiospore production was increased in AmutBmut×5337 approximately 2.8-fold compared to AmutBmut. Basidiospore viability was high in both of these strains. In contrast, line #2 produced a reduced number of basidiospores (16% of the amount produced by AmutBmut). Although #2×5337 produced

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**Table 2. Copy number, by Southern analysis, of constructs inserted in the genome**

The number of constructs inserted in genomic DNA was determined by Southern analysis.

<table>
<thead>
<tr>
<th>Lineage no.</th>
<th>Number of intact constructs</th>
<th>Number of promoter only</th>
<th>Number of terminator only</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>#2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>#11</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>#12</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>#14</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>#20</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>#21</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>#23</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>#35</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>#44</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
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**Fig. 3.** Analysis of pCcLIM15dsRNA insertions in white-cap transformants. Genomic DNA from AmutBmut (A, E), white-cap transformants (B–D, F–H) was digested with the indicated restriction enzymes and hybridized with the indicated probes (β-pro, β-tubulin promoter probe; β-ter, β-tubulin terminator probe. The endogenous loci of the β-tubulin gene are indicated by asterisks (arrow, Apal digestion; arrowhead, HindIII digestion).
4.7-fold more basidiospores than #2, this was still reduced compared with *AmutBmut* (and only 28% of the amount produced by *AmutBmut* × 5337). The basidiospore viability of line #2 (66%) and #2 × 5337 (58%) was lower than that of controls. The basidiospores of line #2 and #2 × 5337 were paler than those of controls (data not shown).

Furthermore, we analysed *LIM15* mRNA expression in heterozygote #2 × 5337 (Fig. 5C). Like line #2, #2 × 5337

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**Fig. 4.** Northern analysis in white cap transformants. Total RNA from *AmutBmut*, spo22-1 and the white-cap transformants was blotted and hybridized with probes as indicated. Twenty micrograms total RNA was blotted in each lane.

**Fig. 5.** Effect of *LIM15* dsRNA in heterozygote. Effect of the *LIM15* dsRNA on the cap colour of the mature fruiting body. (A) *AmutBmut* × 5337 showed normal basidiospore formation. (B) A heterozygote in which one nucleus had *LIM15* dsRNA showed abnormal basidiospore formation and resulted in a grey cap. Each picture was taken the day after sporulation. (C) Northern analysis. Total RNA from *AmutBmut*, line #2, *AmutBmut* × 5337 and #2 × 5337 was blotted and hybridized with probes as indicated. Twenty micrograms total RNA was blotted in each lane. As a *LIM15* probe, we employed the *LIM15* 1–750 nt probe which we used in the construct.
eventually became anucleate cells (33% K cells to contain a condensed nucleus (52%)). Following this discrete stage, we observed these cells containing a condensed nucleus (Fig. 6I) and anucleate cells (indicated by the arrow in Fig. 6I). In line #2 and in #2 x 5337, we rarely observed normal progression of meiosis and sporulation. These phenotypes are common to the other white-cap transformants that show normal progression around karyogamy.

In meiotic prophase I, axial elements (AEs) form as thread-like structures, then AEs become lateral elements (LEs). The synaptonemal complex (SC), a proteinaceous structure between aligned homologous LEs, forms along their lengths. SC formation is a crucial step for normal meiotic progression. To examine the chromosome status in meiotic prophase I precisely, we spread chromosomes from meiotic cells at the pachytene stage of meiotic prophase I, stained them with silver nitrate, and viewed them with an electron microscope. As reported elsewhere (Celerin et al., 2000), AmutBmut showed fully synapsed chromosomes and proper SC formation (Fig. 7A, D). However, although line #2 and #2 x 5337 showed AE formation, they did not show proper synopsis (Fig. 7B, C, E and F). These aberrant features of line #2 and #2 x 5337 were similar to each other. These results indicate that C. cinereus Lim15 plays a role in homologue synopsis after AE formation, as described in other eukaryotic species (Bishop et al., 1992; Yoshida et al., 1998).

**DISCUSSION**

To investigate meiosis in C. cinereus, sporulation-defective mutants have been generated by forward-genetics techniques, including mutagenesis by UV, gamma-irradiation and restriction-enzyme-mediated integration (REMI; reviewed by Casselton & Zolan, 2002). Forward genetics is a powerful tool to make interesting mutants. However, it requires a great deal of labour and time, and it has been impossible to make targeted specific mutants such as LIM15. In this study, we demonstrate the first successful repression of the meiosis-specific recombinase LIM15 in C. cinereus.

We confirmed that the introduction of LIM15dsRNA correlates with specific reduction of the LIM15 transcript. However, all white-cap transformants had multiple insertions of intact and non-intact constructs (Table 2). We cannot completely exclude the possibility of mutagenesis by random insertion of plasmids into the C. cinereus genome. In C. cinereus, transformation is associated with random cutting of DNA and random insertion into the genome (Binninger et al., 1991). We think that this feature is a shortcoming of C. cinereus in terms of evaluating the effect of transformation. To exclude the integration of non-intact constructs, further technical modification of the transformation procedure will be required.

In spite of these caveats, we argue that the effects of our LIM15dsRNA are specific, for four reasons. First, as shown

**Table 3. Basidiospore production and viability**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Basidiospore production</th>
<th>Basidiospore viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmutBmut</td>
<td>1.4 x 10⁴ ± 2 x 10³</td>
<td>96% (n = 321)</td>
</tr>
<tr>
<td>#2</td>
<td>2.3 x 10⁴ ± 2 x 10³</td>
<td>66% (n = 322)</td>
</tr>
<tr>
<td>AmutBmut x 5337</td>
<td>3.9 x 10⁴ ± 4 x 10³</td>
<td>88% (n = 352)</td>
</tr>
<tr>
<td>#2 x 5337</td>
<td>1.1 x 10⁴ ± 6 x 10²</td>
<td>58% (n = 324)</td>
</tr>
</tbody>
</table>

Basidiospore production was counted per milligram of cap tissue.
in Table 1, repression of *LIM15* occurred specifically when we transformed with pCc*LIM15*dsRNA. Second, paucity of *LIM15* mRNA occurred without disturbing the endogenous *LIM15* locus. A previous report has indicated that the frequency of genomic insertion at homologous sites by transformation is approximately 5% in *C. cinereus* (Binninger *et al*., 1991). However, we did not detect any insertion at the homologous site of *LIM15* and at the β-tubulin gene locus in any of our transformants. Third, the trans effect of the construct in the heterozygote might be associated with the action of dsRNA on the other normal allele. This phenotype is different from that of recessive meiotic mutants which show no meiotic phenotype in the heterozygous state. Fourth, the meiosis-specific phenotype in these transformants supports the specific action of *LIM15*dsRNA. Abnormal homologous synapsis is a typical feature of *LIM15*/DMC1 mutants in eukaryotic species (reviewed by Masson & West, 2001).

From these observations, we speculate that an RNAi pathway is likely to exist in *C. cinereus*. The proteins required for the RNAi pathway are known to be conserved in

<table>
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<tr>
<th></th>
<th>Frequency (%)</th>
<th>K+9</th>
<th>K+12</th>
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<tbody>
<tr>
<td></td>
<td>AmutBmut #2</td>
<td>#2 x 5337</td>
<td>AmutBmut #2</td>
</tr>
<tr>
<td>One broad nucleus</td>
<td>0</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Discrete chromosomes</td>
<td>0.7</td>
<td>41.4</td>
<td>55.2</td>
</tr>
<tr>
<td>One condensed nucleus</td>
<td>5.0</td>
<td>52.6</td>
<td>35.1</td>
</tr>
<tr>
<td>Two nuclei</td>
<td>10.8</td>
<td>0</td>
<td>3.0</td>
</tr>
<tr>
<td>Four nuclei</td>
<td>73.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anucleate</td>
<td>16.1</td>
<td>5.1</td>
<td>6.0</td>
</tr>
</tbody>
</table>

**Fig. 6.** Meiotic progression of *LIM15* repression strains. (A–I) The meiotic cells of *AmutBmut*, line #2 and #2 x 5337 were stained with DAPI. The small rectangle in (D) shows an example of cells at meiotic metaphase I. The arrow in panel I indicates an anucleate cell. (J) Frequency of various shapes of meiotic cells at K+9 and K+12. A broad nucleus represents meiotic prophase I, a condensed nucleus represents meiotic metaphase I in normal meiosis. Anucleate cells of *AmutBmut* at K+12 correspond to cells that have undergone sterigmata formation and basidiospore ejection.
various eukaryotic organisms. Using the C. cinereus genomic database (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), we found a Dicer homologue in C. cinereus. Similarly, the C. cinereus genomic database also has a sequence which has homology to the Zw ill/ARGONAUTE/Piwi family that has been implicated in the RNAi pathway in Caen. elegans (RDE-1; Tabara et al., 1999) and Neurospora (QDE-2; Catalanotto et al., 2000). Further analysis of dsRNA-mediated gene silencing in C. cinereus will shed light on the detailed mechanism of RNAi in C. cinereus and in other homobasidiomycete fungi.

Interestingly, although the LIM15 repression strain (line #2) showed a defect in SC formation, a significant amount of basidiospore production and basidiospore viability was still observed. One possible explanation of these results is that Rad51 compensates for the loss of Lim15 function due to redundant function of Rad51 and Lim15. However, we support another explanation: incomplete repression of LIM15 permits a significant amount of sporulation. We could detect faint signals of LIM15 by Northern analysis. This low-level expression might result in occasional normal progression of meiosis and sporulation. These results may indicate that gene silencing by LIM15dsRNA is incomplete.

We propose several further applications of dsRNA-mediated gene silencing in C. cinereus. First, dsRNA-mediated gene silencing can produce partial repression of a gene of interest. This may be useful for the study of essential genes, since incomplete silencing might rescue the lethality and display an intermediate phenotype. Second, dsRNA-mediated gene silencing can be regulated spatially and temporally by dsRNA expression using a specific promoter. For example, we have previously studied DNA-replication-related factors, such as DNA polymerase α and PCNA, during meiosis (Namekawa et al., 2003b; Hamada et al., 2002). Using a meiosis-specific promoter to express the dsRNA of these cDNAs, it will be possible to investigate the function of these genes during meiotic prophase I separately from pre-meiotic S phase or mitotic S phase. Third, we can simultaneously induce repression of multiple genes by introducing multiple dsRNAs. Lastly, future experiments can take advantage of the trans effect of the construct specifically to study whether heterozygoticity is sufficient to induce the phenotype. It may be easy to induce the repression of multiple genes at the dikaryonic stage by making a heterozygote of different dsRNA expression monokaryons.

In these ways, this study opens up doors for the use of the reverse-genetics approach in C. cinereus. The C. cinereus genome project was published in 2003. dsRNA-mediated gene silencing will be an important tool for C. cinereus research in the post-genomic era.

**Fig. 7.** Electron micrographs of meiotic nuclei of LIM15 repression strains. The meiotic nuclei at K+6 (the pachytene stage) of AmutBmut, line #2 and #2 x 5337 were stained with silver nitrate. (D–F) Four-times magnified images of (A–C). The magnified positions are indicated by the arrows.
ACKNOWLEDGEMENTS

We would like to thank D. E. Cohen and M. C. Anguera for critical reading of the manuscript. Y. Kondo for help with the electron microscopy, K. Takata and T. Ishibashi for technical advice about the LIM15 dsRNA expression construct, and P. J. Pukkila and M. E. Zolan for the gift of strains AmutBmut and spo22-1 and for technical guidance for the electron microscopy. S. H. N. was a research fellow of Japan Society for the Promotion of Science.

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