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 synapsis 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.

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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 photo-induction (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 I, 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* (Binnering *et al.*, 1991), so far,

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; PAB, 4-aminobenzoic acid; RNAi, RNA interference; SC, synaptonemal complex.

The results of Southern analysis of pCc*LIM15dsRNA* insertions in white-cap transformants of *C. cinereus* are shown in Supplementary Fig. S1 with the online version of this paper.

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 small 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 *Caenorhabditis 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 recombinase: 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 mushroom cap colour without affecting growth and morphogenesis. 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 synapsis during meiosis. The applications of dsRNA-mediated gene silencing in *C. cinereus* research are discussed.

METHODS

Strains and culture of *C. cinereus*. The *C. cinereus* strains *AmutBmut* (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 extract/yeast extract/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 basidia have fused nuclei, began at 04:00 (K+0), 1 h before the light was turned on; photo-induction started at 05:00 (K+1).

Construction of pCcLIM15dsRNA. The expression vector pCcCEX (*C. cinereus* constitutive expression), which has 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 terminator, was made as follows. A 393 bp fragment of the β -tubulin promoter (Cummings *et al.*, 1999) was cloned into the pCRII vector (Invitrogen) at *Hind*III and *Eco*RI sites. Then, the resulting plasmid was digested with *Eco*RI and *Not*I, and a multi-cloning site containing *Eco*RI, *Sac*II, *Xho*I, *Kpn*I, *Xba*I and *Not*I sites was ligated into the digested site. The 427 bp fragment of the β -tubulin terminator sequence was cloned into the *Not*I and *Apa*I 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 *Xho*I and *Kpn*I sites of pCcCEX. Next, the sense strand which corresponds to 101–750 bp in *LIM15* cDNA was cloned into the *Xba*I and *Not*I sites of the resulting plasmid to generate pCcLIM15dsRNA.

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

Genetic technique for mating to make heterozygotes. The inocula (1 $\times$ 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 basidiospores 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 (Pukkila & 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

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 (*LIM15*dsRNA). Since we used the meiosis-specific transcript *LIM15* as a target, we predicted meiosis-specific defects by the constitutive expression of *LIM15*dsRNA.

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 *LIM15*dsRNA, 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 *LIM15*dsRNA into the genome by Southern analysis (Fig. 3). We extracted genomic DNA from each white-cap

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.

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

transformant and digested it with either *HindIII*, a site for which is located at the 5' end of the β -tubulin promoter, or *ApaI*, a site for which is located at the 3' end of the β -tubulin terminator (Fig. 1), using either the β -tubulin promoter probe or the β -tubulin terminator probe. If the entire *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 β -tubulin locus. For example, line #2 displayed one such band (indicated by an asterisk), in addition to the endogenous β -tubulin bands in Fig. 3, lanes 6–9. When *HindIII* 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 *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 *LIM15*dsRNA has the β -tubulin promoter and terminator, the endogenous bands from the β -tubulin were unchanged in all cases (Fig. 3A–D and data not shown). These results indicate that the β -tubulin locus was not disturbed by homologous recombination in these white-cap transformants. Further Southern analysis indicated that the endogenous band of *LIM15* was unchanged (Fig. 3E–F and Supplementary Fig. S1). This confirmed that transformation did not alter the endogenous *LIM15* gene locus by homologous recombination.

To investigate whether endogenous *LIM15* is repressed in white-cap transformants, we performed Northern analysis. Our previous results demonstrated that the expression of *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 *LIM15* transcript compared to *AmutBmut* and the recessive meiotic mutant *spo22-1* (Kanda *et al.*, 1990). Similar results were obtained with the *LIM15* 1–750 nt probe, which contains the sequence from our dsRNA, and the *LIM15* 801–1041 nt probe, which does not. The same filter was hybridized with *RAD51* cDNA, which has high sequence identity to *LIM15* (Nara *et al.*, 1999). A significant difference was not observed between the pattern of *RAD51* and the

Table 1. Constructs used for *LIM15* gene silencing and their effect on cap colour

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

Plasmid (marker)	Plasmid (construct)	Number of clones tested	Number with black cap	Number with white cap
pPAB1-2	—	27	27	0
pPAB1-2	pCcCEX	48	48	0
pPAB1-2	pCc <i>LIM15</i> dsRNA	44	34	10

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).

constitutively expressed gene *G3PDH*. Taken together, these results indicate that the entire *LIM15* coding sequence was specifically repressed by the transformation of *LIM15*dsRNA.

trans Effect of *LIM15*dsRNA 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 *LIM15*dsRNA 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 *LIM15*dsRNA, we mated wild-type monokaryotic strain 5337 (Murata *et al.*, 1998), with line #2, and produced dikaryotic strain #2 $\times$ 5337. #2 $\times$ 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* $\times$ 5337. *AmutBmut* $\times$ 5337 showed normal meiosis (Fig. 5A).

To compare the effect of *LIM15*dsRNA in these strains, we determined the basidiospore production and viability (Table 3). As shown in Table 3, basidiospore production was increased in *AmutBmut* $\times$ 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 $\times$ 5337 produced

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.

Lineage no.	Number of intact constructs	Number promoter only	Number terminator only
#1	2	4	2
#2	1	1	0
#11	3	4	1
#12	2	2	1
#14	4	1	1
#20	1	2	1
#21	2	1	3
#23	1	4	2
#35	1	2	2
#44	4	2	2

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.

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

Fig. 5. Effect of *LIM15dsRNA* in heterozygote. Effect of the *LIM15dsRNA* on the cap colour of the mature fruiting body. (A) *AmutBmut* × 5337 showed normal basidiospore formation. (B) A heterozygote in which one nucleus had *LIM15dsRNA* 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.

Table 3. Basidiospore production and viability

Basidiospore production was counted per milligram of cap tissue.

Strain	Basidiospore production	Basidiospore viability
<i>AmutBmut</i>	$1.4 \times 10^4 \pm 2 \times 10^3$	96 % ($n=321$)
#2	$2.3 \times 10^3 \pm 2 \times 10^3$	66 % ($n=322$)
<i>AmutBmut</i> × 5337	$3.9 \times 10^4 \pm 4 \times 10^3$	88 % ($n=352$)
#2 × 5337	$1.1 \times 10^4 \pm 6 \times 10^2$	58 % ($n=324$)

shows very low levels of *LIM15* without reducing Rad51 expression. Taking all these results into account, the heterozygote (#2 × 5337) shows a similar phenotype to the homozygote (line #2). These results indicate that *LIM15*dsRNA exercises a *trans* effect on the other nucleus in the heterozygote.

We examined whether the phenotype survived sporulation. We obtained spores from transformant #2, from which mycelium was cultured, and 100 % of spores ($n=7$) displayed a white cap. This result indicated that the phenotype was heritable.

Meiotic phenotype of *LIM15* repression strain

To investigate the meiotic defect in white-cap transformants, we examined meiotic chromosome behaviour using DAPI, and compared meiotic chromosomes from line #2 with chromosomes from *AmutBmut*. We defined the time of karyogamy (K) to be the time at which 5 % of cells undergo nuclear fusion. In the wild-type, nuclear fusion continues for some hours after karyogamy, with almost all nuclei being fused by K + 4. Subsequently, meiotic prophase I can be observed in single nuclei until K + 7. Meiotic prophase I is followed by meiosis I and meiosis II, and results in tetrads at K + 12.

Consistent with previous reports (Celerin *et al.*, 2000), we found that *AmutBmut* followed this standard progression. We observed one broad nucleus per cell at meiotic prophase I (K + 4; Fig. 6A), followed by the first and second divisions of meiosis at K + 9 (Fig. 6D). Tetrad and beetling sterigmata formation, which will become the basidiospore after nuclear migration, was also observed in normal basidiospore development (Fig. 6G). *AmutBmut* × 5337 also showed normal progression of meiosis (data not shown). In contrast, although line #2 shows one broad nucleus per cell with meiotic prophase I proceeding normally (Fig. 6B), the majority of cells of line #2 could not divide into two nuclei, and meiosis did not proceed beyond meiosis I. Discrete chromosomes were observed in the nucleus in K + 9, indicative of abnormal meiosis (41.4 %, $n=175$; Fig. 6E, J). Following this discrete stage, we observed these cells to contain a condensed nucleus (52.6 %, $n=175$ at K + 9; 43.2 %, $n=81$ at K + 12; Fig. 6E, H and J), and they eventually became anucleate cells (33.3 %, $n=81$ at K + 12; Fig. 6H, J). This suggested that line #2 arrested at meiotic

metaphase I. Chromatin condensation and anucleate cells are common features in all meiotic mutants of *C. cinereus* (Lu *et al.*, 2003). Also, in #2 × 5337, cells showed discrete chromosomes at K + 9 (55.2 %, $n=134$; Fig. 6F, J). By K + 12, we observed cells containing a condensed nucleus (Fig. 6I) and anucleate cells (indicated by the arrow in Fig. 6I). In line #2 and in #2 × 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 × 5337 showed AE formation, they did not show proper synapsis (Fig. 7B, C, E and F). These aberrant features of line #2 and #2 × 5337 were similar to each other. These results indicate that *C. cinereus* Lim15 plays a role in homologue synapsis 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 *LIM15*dsRNA 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 *LIM15*dsRNA are specific, for four reasons. First, as shown

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.

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

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 × 5337 were stained with silver nitrate. (D–F) Four-times magnified images of (A–C). The magnified positions are indicated by the arrows.

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 Zwill/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 *LIM15*dsRNA 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 heterozygosity is sufficient to induce the phenotype. It may be easy to induce the repression of multiple genes at the dikaryotic 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.

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