Molecular breeding of sporeless strains of *Pleurotus ostreatus* using a non-homologous DNA end-joining defective strain

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Abstract

Gene targeting is useful to isolate strains with mutations in a gene of interest for efficient breeding. In this study, we generated *msh4* or *mer3* single-gene disruptant monokaryons using a *Pleurotus ostreatus* $\Delta ku80$ strain for efficient gene targeting. Dikaryons of *P. ostreatus* $\Delta msh4 \times \Delta msh4$ or $\Delta mer3 \times \Delta mer3$ were isolated via backcrosses, and the number of basidiospores produced was measured. The number of basidiospores fell by an average 1/13.7 in the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ dikaryons versus the *P. ostreatus* *msh4*⁺ $\times \Delta msh4$ dikaryons, and 1/82.6 in the *P. ostreatus* $\Delta mer3 \times \Delta mer3$ dikaryons versus the *P. ostreatus* *mer3*⁺ $\times \Delta mer3$ dikaryons. To demonstrate the effects of *ku80* disruption, *P. ostreatus* $\Delta ku80 \times \Delta ku80$ dikaryon strains were isolated and no significant effects on basidiospore production were observed. Fluorescence microscopy showed meiotic progression was arrested during prophase I in the *msh4* or *mer3* disruptants. To our knowledge, this is the first report on molecular breeding of sporeless strains in cultivated mushrooms using an efficient method for targeted gene disruption.

Keywords *Pleurotus ostreatus* · *msh4* · *mer3* · Sporeless · Meiosis

Introduction

Molecular breeding is a promising breeding strategy because one can isolate strains with desired phenotypes directly. Gene targeting is especially powerful for isolating strains with modifications in a gene of interest for efficient breeding purposes. Moreover, it is also effective to characterize the gene function for research purposes. However, the targeted molecular breeding of a cultivated mushroom strain is not common because of the low gene-targeting efficiency.

An efficient technique for gene targeting by homologous recombination was established in filamentous fungi by disrupting one or two of the genes encoding Ku70/Ku80 and Ligase 4 (Ruiz-Díez 2002; Ninomiya et al. 2004; Levy et al. 2008; de Jong et al. 2010; Kück and Hoff 2010; Nakazawa et al. 2011). These proteins play a major role in the non-homologous DNA end-joining (NHEJ) system, and their

inactivation makes homologous recombination the relatively dominant repair system in the mycelia, which leads to the establishment of high-frequency gene targeting. The oyster mushroom *Pleurotus ostreatus* is one of the most economically important edible mushrooms in the world, and an efficient gene targeting was developed for the first time in the cultivated mushrooms by isolating a *P. ostreatus* $\Delta ku80$ monokaryon (Salame et al. 2012).

A strain with low production of basidiospores is an important breeding target in the mushroom industry as the dispersal of basidiospores into cultivation facilities causes allergic reactions in workers/handlers as well as facility disruption (Baars et al. 2000). The *msh4* and *mer3* genes encode proteins required for meiosis (Snowden et al. 2004; Sugawara et al. 2009), and their inactivation is reported to cause a deficiency in spore formation in *Coprinopsis cinerea* and *Pleurotus pulmonarius* (Sugawara et al. 2009; Okuda et al. 2013). In this study, we generated *msh4* or *mer3* disruptants by gene knock out using the $\Delta ku80$ strain of *P. ostreatus* and analyzed the effects on sporulation to demonstrate targeted molecular breeding of cultivated mushrooms.

Furthermore, it was suggested that NHEJ might be involved in the meiotic process of eukaryotes such as mouse and *Coprinopsis cinerea* (Namekawa et al. 2003; Liebe et al. 2006; Okuda et al. 2013). Disruption of *ku80* may cause

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defects in sporulation in *P. ostreatus*; therefore, the effects of *ku80* disruption on basidiospore production were also analyzed.

Materials and methods

Strains, culture conditions, and genetic techniques

P. ostreatus strains used in this study are listed in Table 1. Yeast and malt extract with glucose (YMG) medium (Rao and Niederpruem 1969) solidified with 2% (w/v) agar in 9-cm Petri dishes was used for routine cultures. The strains were grown at 28 °C under continuous darkness. The cultures were maintained at 4 °C for long-term storage. Dikaryotic strains of *P. ostreatus* were also grown on sawdust (*Fagus crenata*) media with 7.5% (w/w) wheat bran supplementation to induce fruiting body formation as described by Nakazawa et al. (2016). Sawdust and wheat bran were purchased from Shinkoen (Gifu, Japan) and Nisshin Seifun (Tokyo, Japan). Crosses and fruiting of *P. ostreatus* were performed as described by Inada et al. (2001) and Nakazawa et al. (2016), respectively. Dikaryon formation was confirmed by observation of clamp cells 6 days after mating.

Construction of *msh4*- and *mer3*-disrupting plasmids

To generate a *msh4*-disrupting plasmid, upstream (1477 bp) and downstream (1452 bp) fragments of the *msh4* gene, corresponding to Protein ID 65320 [*msh4*; Scaffold_19: 5841–9704 of *P. ostreatus* PC9 (https://mycocosm.jgi.doe.gov/PleosPC9_1/PleosPC9_1.home.html)], were amplified using a primer pair FY18/FY19 and FY20/FY21 (Table 2) from *P. ostreatus* 20b. A genomic fragment containing the hygromycin-B-resistance gene (*hph*) was also amplified using primers TN400/M13R from pTN24-1 (Nakazawa et al. 2017b). These three fragments were fused by overlap-extension polymerase chain reaction (PCR). The resulting *msh4*-disrupting cassette was cloned into pBluescript II KS+ digested with *EcoRV*, yielding pBS- Δ *msh4* (Fig. 1a).

To generate a *mer3*-disrupting plasmid, a genomic fragment of the *mer3* gene, corresponding to Protein ID 82484 [*mer3*; Scaffold_1: 2926299–2932521 of *P. ostreatus* PC9], was amplified using a primer pair FY31/FY34 and cloned into pBluescript II KS+ digested with *EcoRV*. Inverse PCR was performed using a primer pair FY32/FY33. A genomic fragment containing the hygromycin-B-resistance gene was also amplified using a primer pair TN400/M13R from pTN24-1. These two DNA fragments were fused using the Geneart Seamless Cloning and Assembly kit (Life Technologies, CA, USA). The resulting plasmid containing a *mer3*-disrupting cassette was designated as pBS- Δ *mer3* (Fig. 1b). The cassettes were amplified from pBS- Δ *msh4* and

pBS- Δ *mer3* using a primer pair FY18/FY21 and FY31/FY34, respectively, when used for transformation.

PCR experiments were performed using a KOD FX Neo (Toyobo, Japan) with the following program: an initial denaturing step at 94 °C for 2 min, followed by 35 cycles of 10 s at 98 °C, 30 s at 58 °C, and 30 s/kb at 68 °C, and a final extension step at 68 °C for 5 min.

Isolation of *msh4* or *mer3* single-gene disruptants

A mixture of pBS- Δ *msh4* and *msh4*-disrupting cassette (about 2 μ g and 1 μ g, respectively) was introduced into protoplasts of *P. ostreatus* 20b (about 5×10^7) to obtain the disruptant of *msh4*. A mixture of pBS- Δ *mer3* and *mer3*-disrupting cassette (about 2 μ g and 1 μ g, respectively) was also introduced into protoplasts of *P. ostreatus* 20b (about 5×10^7) to obtain the disruptant of *mer3*. The hygromycin resistance transformation of *P. ostreatus* 20b, a Δ *ku80* derivative of *P. ostreatus* PC9, was performed using protoplasts prepared from mycelial cells as described by Salame et al. (2012), except that heparin and single-strand lambda phage DNA were not used as described by Nakazawa et al. (2016).

Extraction of genomic DNA and PCR

Genomic DNA was extracted from each strain as described by Tsukihara et al. (2006) and Nakazawa et al. (2016), followed by genomic PCR using an EmeraldAmp MAX PCR Master Mix (TAKARA BIO, Japan). The program was 35 cycles of 10 s at 94 °C, 30 s at 58 °C, and 1 min/kb at 72 °C.

Basidiospore measurement

Fruiting bodies of each dikaryotic strain were formed on plastic-bottled sawdust media (Nakazawa et al. 2019). Bottles with fruiting bodies were placed on the center of empty Petri dishes. After 1 day (Table 3) or 3 days (Table 4), basidiospores falling from the fruiting body onto the Petri dish were collected in a 1.5-mL tube using 1 mL water. After that, the number of basidiospores was counted using a hemocytometer.

Fluorescence microscopy

For observation of basidia, gills were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.5) for 2 h. Next, samples were stained with 4,6-diamidino-2-phenylindole (DAPI) after washing several times with PBS and observed under an AxioScope. A1 fluorescence microscope (ZEISS, Germany) equipped with a filter set 91 HE.

Table 1 *P. ostreatus* strains used in this study

Strain	Genotype/description	Source	No. of basidiospores ($\times 10^6$)
PC9	A2B1	Laraya et al. (1999)	
PC15	A1B2	Laraya et al. (1999)	
#64	A64B64	Nakazawa et al. (2017a)	
20b	A2B1 ku80::Cbx ^R ^a	Salame et al. (2012)	
20b×PC15	A2B1 A1B2	This study	
20b×#64	A2B1 A64B64	This study	
$\Delta msh4\#1$	A2B1 ku80::Cbx ^R msh4::hyg ^R ^b / a msh4 disruptant derived from 20b	This study	
$\Delta mer3\#1$	A2B1 ku80::Cbx ^R mer3::hyg ^R / a mer3 disruptant derived from 20b	This study	
msh4 ⁺ × $\Delta msh4\#1$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^S F ₁ strain from $\Delta msh4\#1$ and #64	This study	880
msh4 ⁺ × $\Delta msh4\#2$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^S F ₁ strain from $\Delta msh4\#1$ and #64	This study	112
msh4 ⁺ × $\Delta msh4\#3$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^S F ₁ strain from $\Delta msh4\#1$ and #64	This study	370
$\Delta msh4 \times \Delta msh4\#1$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^R F ₁ strain from $\Delta msh4\#1$ and #64	This study	0.1
$\Delta msh4 \times \Delta msh4\#2$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^R F ₁ strain from $\Delta msh4\#1$ and #64	This study	132
$\Delta msh4 \times \Delta msh4\#3$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^R F ₁ strain from $\Delta msh4\#1$ and #64	This study	0
$\Delta msh4 \times \Delta msh4\#4$	A2B1 A64B64 / a cross between $\Delta msh4\#1$ and the hyg ^R F ₁ strain from $\Delta msh4\#1$ and #64	This study	0.9
mer3 ⁺ × $\Delta mer3\#1$	A2B1 A1B2 / a cross between $\Delta mer3\#1$ and the hyg ^S F ₁ strain from $\Delta mer3\#1$ and PC15	This study	188
mer3 ⁺ × $\Delta mer3\#2$	A2B1 A64B64 / a cross between $\Delta mer3\#1$ and the hyg ^S F ₁ strain from $\Delta mer3\#1$ and #64	This study	496
$\Delta mer3 \times \Delta mer3\#1$	A2B1 A1B2 / a cross between $\Delta mer3\#1$ and the hyg ^R F ₁ strain from $\Delta mer3\#1$ and PC15	This study	0
$\Delta mer3 \times \Delta mer3\#2$	A2B1 A64B64 / a cross between $\Delta mer3\#1$ and the hyg ^R F ₁ strain from $\Delta mer3\#1$ and #64	This study	5
$\Delta mer3 \times \Delta mer3\#3$	A2B1 A64B64 / a cross between $\Delta mer3\#1$ and the hyg ^R F ₁ strain from $\Delta mer3\#1$ and #64	This study	0.1
$\Delta mer3 \times \Delta mer3\#4$	A2B1 A64B64 / a cross between $\Delta mer3\#1$ and the hyg ^R F ₁ strain from $\Delta mer3\#1$ and #64	This study	7.2
$\Delta mer3 \times \Delta mer3\#5$	A2B1 A64B64 / a cross between $\Delta mer3\#1$ and the hyg ^R F ₁ strain from $\Delta mer3\#1$ and #64	This study	8.4
m4#1	A64B64 ku80::Cbx ^R msh4::hyg ^R / the F ₁ strain from $\Delta msh4\#1$ and #64	This study	

^a The carboxin-resistance marker^b hyg^S and hyg^R indicate hygromycin-sensitive and -resistant, respectively

Table 2 The primers used in this study

Primer	Sequence (5' - 3')
FY9	TTCAAGCATTACGACGGTACC
FY14	AGACGTACCTGAGGTCCTGTC
FY15	AACCCCAAGTTCTATGCGTTG
FY16	ACTTACTCTTCTGCAGCCGAC
FY17	TACTCGTCAGCTCCGTACTC
FY18	ACCTTCCTTGACGGCTGGAC
FY19	ACGTCGTGACTGGGATTAAGAACAGGAGAAAGCGTG
FY20	CCTGTGTGAAATTGTTGGGCCTCAGGCTGGTATAAAG
FY21	ATAGTATTCGGAACAGACCGGAC
FY22	TTAGTAATAGGCGCATCCTGC
FY23	AGTTCACCGATATCCGCGATG
FY24	TCTTGTTTGCCTAACACATATTG
FY31	TGCTCGTGCTTGTGCTCACTG
FY32	ACGTCGTGACTGGGATTGGAACAGGAATGAACCGC
FY33	CCTGTGTGAAATTGTAGCTCATGCTCCCAGATTCTCTG
FY34	TGACAACCGCAGGCTTCAGGTC
M13R	ACAATTTACACAGGAAACAGCTATGACC
TN48	TGGCTGTTGACTTATCGTGCGC
TN378	TCACAAGTTAACAGCCACGGATTAAGC
TN400	TCCCAGTCACGACGTCAGGCAAAACCCAGATACGC

Results

Generation of *msh4* or *mer3* disruptants from *P. ostreatus* strain 20b

Approximately 10 hygromycin-resistant $\Delta msh4$ or $\Delta mer3$ transformants were obtained after the transformation of *P. ostreatus* 20b with the respective disruption cassette/plasmid mixture. PCR-amplifications using primer sets A–D were used to determine whether *msh4* or *mer3* gene had been replaced with the respective *hph* cassette. This way, for the $\Delta msh4$ transformants, disruption of $\Delta msh4$ could be proven in one out of one transformant, and for the $\Delta mer3$ transformants, disruption of $\Delta mer3$ could be proven in three out of five transformants (Fig. 1c, d, e, and f). Primer sets A and B yielded amplicons from the gene disruptants, but not from *P. ostreatus* 20b. Primer set C produced an amplicon from *P. ostreatus* 20b, but not from the gene disruptants. As shown in Fig. 1c and e, the fragment was amplified from the transformant when primer set A (FY17/TN48) or B (TN378/FY22) was used, but not when primer set C (FY23/FY24) was used. In addition, it was expected that a 6864-bp genomic fragment was PCR-amplified from *P. ostreatus* 20b and 5332-bp from the transformant when primer set D (FY17/FY22) was used (Fig. 1c). As shown in Fig. 1e, the expected results were obtained from this genomic PCR experiment, although the possibility of the ectopic

integration of the *hph* cassette could not be ruled out. These results indicate that the *msh4* gene is replaced with the *hph* cassette in the transformant, which was designated as *P. ostreatus* $\Delta msh4\#1$. Similarly, the *mer3* gene was shown to be disrupted in three out of the five transformants. PCR results for the *mer3* disruptant, *P. ostreatus* $\Delta mer3\#1$, are shown in Fig. 1d and f.

Fig. 1 Targeted disruption of *msh4* or *mer3*. **a** A diagram illustrating the procedure for the construction of pBS- $\Delta msh4$. The red box indicates the 5' region (1477 bp), and green the 3' region (1452 bp) of the *msh4* gene. The sequence of *msh4*-disrupting cassette is shown in Online Resource 1. **b** A diagram illustrating the procedure for the construction of pBS- $\Delta mer3$. The red box indicates the 5'-side ORF of the *mer3* gene (1539 bp), and the green one the 3'-side ORF of the *mer3* gene (1525 bp). The sequence of *mer3*-disrupting cassette is shown in Online Resource 2. **c** A diagram of the genomic locus of *msh4*. Arrows indicate the primers used for the PCR experiments in e. **d** A diagram of the genomic locus of *mer3*. Arrows indicate the primers used for the PCR experiments in f. **e** PCR experiments confirming *msh4* disruption. Lane 1, negative control (genomic DNA was not added); Lane 2, *P. ostreatus* 20b; Lane 3, *P. ostreatus* $\Delta msh4\#1$; and Lanes M represent size markers: λ HindIII marker on the upper, Ladder marker on the lower left, and another Ladder marker on the lower right. **f** Genomic PCR experiments confirming the *mer3* disruption. Lane 1, negative control (genomic DNA was not added); Lane 2, *P. ostreatus* 20b; Lane 3, *P. ostreatus* $\Delta mer3\#1$; and Lanes M on the left and right sides represent size markers: λ HindIII marker on the upper, Ladder marker on the lower left, and another Ladder marker on the lower right

Table 3 Effects of *msh4* or *mer3* disruption on basidiospore production

Strain	No. of basidiospores ($\times 10^6$) ^a	No. of examined strains
20b $\times$ PC15	844 $\pm$ 545	5
20b $\times$ #64	824 $\pm$ 394	5
<i>msh4</i> ⁺ $\times$ Δ <i>msh4</i>	454 $\pm$ 319	3 ^{b, c}
Δ <i>msh4</i> $\times$ Δ <i>msh4</i>	33.3 $\pm$ 57.0	4 ^{b, d}
<i>mer3</i> ⁺ $\times$ Δ <i>mer3</i>	342 $\pm$ 154	2 ^{b, e}
Δ <i>mer3</i> $\times$ Δ <i>mer3</i>	4.14 $\pm$ 3.51	5 ^{b, f}

^a Values indicate means $\pm$ standard deviations^b The number of basidiospores was measured once per each strain^c Three dikaryotic strains: *msh4*⁺ $\times$ Δ *msh4*#1–#3 (Table 1)^d Four dikaryotic strains: Δ *msh4* $\times$ Δ *msh4*#1–#4 (Table 1)^e Two dikaryotic strains: *mer3*⁺ $\times$ Δ *mer3*#1, #2 (Table 1)^f Five dikaryotic strains: Δ *mer3* $\times$ Δ *mer3*#1–#5 (Table 1)

Effects of *msh4* or *mer3* disruption on basidiospore production in *P. ostreatus*

Dikaryotic strains *P. ostreatus* Δ *msh4* $\times$ Δ *msh4*, *msh4*⁺ $\times$ Δ *msh4*, Δ *mer3* $\times$ Δ *mer3*, and *mer3*⁺ $\times$ Δ *mer3* were obtained to measure the number of basidiospores. To create the dikaryons, *P. ostreatus* Δ *msh4*#1 was crossed with *P. ostreatus* #64, and *P. ostreatus* Δ *mer3*#1 was crossed with *P. ostreatus* PC15 or *P. ostreatus* #64 (Table 1). F₁ strains were obtained from each cross (*P. ostreatus* Δ *msh4*#1 $\times$ #64, Δ *mer3*#1 $\times$ PC15, and Δ *mer3*#1 $\times$ #64). F₁ progeny exhibiting resistance to hygromycin B (hyg^R) are considered as Δ *msh4* or Δ *mer3*, and those that were sensitive to hygromycin B (hyg^S) as *msh4*⁺ or *mer3*⁺. The F₁ strains were mated with *P. ostreatus* Δ *msh4*#1 or Δ *mer3*#1 to generate dikaryons (Table 1; three *P. ostreatus* *msh4*⁺ $\times$ Δ *msh4*, four Δ *msh4* $\times$ Δ *msh4*, two *mer3*⁺ $\times$ Δ *mer3*, and five Δ *mer3* $\times$ Δ *mer3*). The number of basidiospores produced by fruiting bodies from the resulting dikaryotic strains was measured to analyze the effects of *msh4* and *mer3* disruption. The number of basidiospores fell by an average 1/13.7 in the *P. ostreatus* Δ *msh4* $\times$ Δ *msh4* dikaryons versus the *P. ostreatus* *msh4*⁺ $\times$ Δ *msh4* dikaryons, and 1/82.6 in the

P. ostreatus Δ *mer3* $\times$ Δ *mer3* dikaryons versus the *P. ostreatus* *mer3*⁺ $\times$ Δ *mer3* dikaryons (Table 3).

To determine the significance of these differences, a statistical analysis using the two-tailed *t* test was performed. The difference between *P. ostreatus* *mer3*⁺ $\times$ Δ *mer3* and Δ *mer3* $\times$ Δ *mer3* was shown to be significant (*P* = 0.0090). In the case of *msh4*, the difference was not significant (*P* = 0.081). The number of basidiospores produced by one of the four strains tested in this study (*P. ostreatus* Δ *msh4* $\times$ Δ *msh4*#2) was 1.32×10^8 and significantly differed from the other *P. ostreatus* Δ *msh4* $\times$ Δ *msh4* strains.

Disruption of *ku80* does not cause a decrease in the number of basidiospores in *P. ostreatus*

Effects of *ku80* disruption on basidiospore production were also analyzed using the F₁ progeny obtained from *P. ostreatus* Δ *msh4*#1 $\times$ PC15 and *P. ostreatus* Δ *msh4*#1 $\times$ #64. F₁ progeny exhibiting resistance to carboxin (cbx^R) are considered Δ *ku80*, and those sensitive to carboxin (cbx^S) as *ku80*⁺. We attempted to obtain F₁ progeny with A64B64, cbx^R and hyg^S backgrounds from *P. ostreatus* Δ *msh4*#1 $\times$ #64; however, we could not. Therefore, a monokaryon with A64B64, cbx^R and

Table 4 Effects of *ku80* disruption on basidiospore production

Strain	No. of basidiospores ($\times 10^6$) ^a	No. of examined strains
20b $\times$ PC15	759 $\pm$ 613	10
20b $\times$ #64	803 $\pm$ 979	9
Δ <i>ku80</i> Δ <i>msh4</i> $\times$ <i>ku80</i> ⁺ <i>msh4</i> ⁺	718 $\pm$ 479	20 ^{b, c}
Δ <i>ku80</i> Δ <i>msh4</i> $\times$ Δ <i>ku80</i> <i>msh4</i> ⁺	1200 $\pm$ 681	5 ^{b, d}

^a Values indicate means $\pm$ standard deviations^b The number of basidiospores was measured once per each strain^c 20 dikaryotic strains: Δ *ku80* Δ *msh4* $\times$ *ku80*⁺ *msh4*⁺ #1–#20^d Five dikaryotic strains: Δ *ku80* Δ *msh4* $\times$ Δ *ku80* *msh4*⁺ #1–#5

hyg^R backgrounds, which is designated as *P. ostreatus* m4#1 (Table 1), was used in the following experiments. *P. ostreatus* m4#1 was mated with 20 cbx^S/hyg^S and five cbx^R/hyg^S F₁ strains from *P. ostreatus* $\Delta msh4\#1 \times PC15$ (the former ones were designated as *P. ostreatus* $\Delta ku80\Delta msh4 \times ku80^+ msh4\#1\text{--}\#20$ and the latter were designated *P. ostreatus* $\Delta ku80\Delta msh4 \times \Delta ku80 msh4\#1\text{--}\#5$). As shown in Table 4, there was no significant difference in the number of basidiospores between *P. ostreatus* $ku80^+ \times \Delta ku80$ and $\Delta ku80 \times \Delta ku80$ ($P=0.092$), suggesting that disruption of *ku80* does not cause significant defects in *P. ostreatus* sporulation.

Meiotic progression is arrested during prophase I in the *msh4* and *mer3* single-gene disruptants

To observe meiotic progress in basidia, the gills were stained with DAPI and observed under a fluorescence microscope. The meiotic stages in a basidium are roughly divided into four stages in *C. cinerea*, a model agaric related to *P. ostreatus*: karyogamy, meiosis I, meiosis II, and sporulation (Fig. 2a; Sugawara et al. 2009). Basidia with tetrad nuclei at telophase

II (Fig. 2b: 1, 2, and 5) and those with two nuclei at telophase I were observed in the *P. ostreatus* 20b $\times$ PC15, $msh4^+ \times \Delta msh4$, and $mer3^+ \times \Delta mer3$ strains, but not in those of *P. ostreatus* $\Delta msh4 \times \Delta msh4$ and $\Delta mer3 \times \Delta mer3$ ones. In the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ and $\Delta mer3 \times \Delta mer3$ strains, basidia with one large nucleus at prophase I (Fig. 2b: 3 and 6), which were not observed in the *P. ostreatus* 20b $\times$ PC15, $msh4^+ \times \Delta msh4$, and $mer3^+ \times \Delta mer3$ strains, and those with no fluorescence after DAPI staining (Fig. 2b: 4 and 7) were observed.

Discussion

Strains defective in the sporulation processes have been reported in other agaricomycetes. In *Pleurotus pulmonarius*, mutations in *stpp1*, an *msh4* homolog, meiotic progression was arrested during prophase I, and basidiospore production reduced to less than 1/1000 of the wild-type control (Okuda et al. 2013). In *C. cinerea*, the expression of *mer3*, which encodes a putative protein involved in the formation of meiotic crossover (Nakagawa and Ogawa 1999), was suppressed

Fig. 2 Meiosis of *P. ostreatus* $\Delta msh4 \times \Delta msh4$ and $\Delta mer3 \times \Delta mer3$ basidia arrested at prophase I. **a** Four stages in meiosis of basidium: karyogamy, meiosis I, meiosis II, and sporulation (Sugawara et al. 2009). **b** Observation of basidia by fluorescence microscope. The scale bar represents 10 μ m. 1: the wild type is *P. ostreatus* 20b $\times$ PC15. Normal tetrad nuclei in the basidium. 2: normal tetrad nuclei in a basidium of the *P. ostreatus* $msh4^+ \times \Delta msh4$. 3: one large nucleus in a basidium of the *P. ostreatus* $\Delta msh4 \times \Delta msh4$. 4: no nuclei in a basidium of the *P. ostreatus* $\Delta msh4 \times \Delta msh4$. 5: normal tetrad nuclei in a basidium of the *P. ostreatus* $mer3^+ \times \Delta mer3$. 6: one large nucleus in a basidium of the *P. ostreatus* $\Delta mer3 \times \Delta mer3$. 7: no nuclei in a basidium of the *P. ostreatus* $\Delta mer3 \times \Delta mer3$

by RNAi. As a result, meiosis progressed only to nuclear fusion, and then apoptosis was observed, and the *mer3*-suppressed homokaryotic strain had a basidiospore production of 1/125 compared with the wild-type strain (Sugawara et al. 2009).

In this study, the number of basidiospores fell by an average 1/13.7 in the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ dikaryons versus the *P. ostreatus* *msh4*⁺ $\times \Delta msh4$ dikaryons, and 1/82.6 in the *P. ostreatus* $\Delta mer3 \times \Delta mer3$ dikaryons versus the *P. ostreatus* *mer3*⁺ $\times \Delta mer3$ dikaryons (Table 3). However, the *t* test indicated that the effect of *msh4* was not significant. These results suggest that both single-gene disruptions of *mer3* and *msh4* cause negative effects on sporulation in *P. ostreatus*, and Mer3 plays more crucial roles than Msh4 does as constant reduction in the numbers of basidiospores from different *P. ostreatus* $\Delta mer3 \times \Delta mer3$ strains whereas the numbers were reduced but varied widely in the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ strains possibly depending on their genetic backgrounds.

Basidia harboring two nuclei were not observed in the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ and $\Delta mer3 \times \Delta mer3$ strains. Moreover, one large nucleus was observed in some of these basidia (Fig. 2b: 3 and 6). These results suggest that an inactivation of *msh4* or *mer3* causes a defect of basidiospore formation in *P. ostreatus* due to arrest of meiotic progression during prophase I. A similar effect was reported in *Schizophyllum commune* mutants with constitutive activation of Ras1 (Knabe et al. 2013). Therefore, examining and comparing the effects of the constitutive activation of Ras1 in *P. ostreatus* to those in *S. commune* would be interesting to study in the future. Basidia without nuclei were also observed in the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ and $\Delta mer3 \times \Delta mer3$ strains (Fig. 2b: 4 and 7). In the case of *C. cinereal*, where meiotic progression is synchronized (Kües 2000), apoptosis of fused nuclei was observed in basidia over time (Sugawara et al. 2009). However, it is not easy to distinguish whether the basidium without a nucleus is the result of nuclear apoptosis or the dispersal of sound basidiospores of *P. ostreatus*; since meiosis is asynchronous in *P. ostreatus*, basidia with various meiotic stages, but not a specific one, were observed at a certain time. However, taking together with very low basidiospore production in the *P. ostreatus* $\Delta msh4 \times \Delta msh4$ and $\Delta mer3 \times \Delta mer3$ strains, apoptosis of nuclei may occur in the meiosis arrested basidia in this fungus.

A few basidiospores were produced in some *P. ostreatus* $\Delta msh4 \times \Delta msh4$ or $\Delta mer3 \times \Delta mer3$ strains, indicating that basidiospore formation is not impaired completely, but maybe significantly delayed in these strains due to the defective meiosis. It is of interest whether double-gene disruption of *msh4* and *mer3* would completely inhibit basidiospore formation in this fungus.

In mice, it was reported that defects in NHEJ affect early prophase I in the meiosis (Liebe et al. 2006). In *C. cinereal*, it was indicated that Lig4 was expressed not only at the pre-meiotic S phase but also at meiotic prophase I (Namekawa et al. 2003). Considering these studies, NHEJ might have a function in the meiotic process in these organisms. However, in the present study, there was no significant difference in the number of basidiospores between *P. ostreatus* *ku80*⁺ $\times \Delta ku80$ and $\Delta ku80 \times \Delta ku80$ dikaryons (Table 4), suggesting that disruption of *ku80* does not cause significant defects in sporulation in *P. ostreatus*.

In conclusion, strains with low production of spores were successfully generated using gene targeting in *P. ostreatus*. The *msh4* and *mer3* disruptant strains isolated in this study showed impaired meiosis that may cause low production of basidiospores. It is also demonstrated that disruption of *ku80* has a negligible effect on basidiospore production in this fungus.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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