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

Fuga Yamasaki1 · Takehito Nakazawa1 · Masahiro Sakamoto1 · Yoichi Honda1

Received: 18 May 2020 / Revised: 26 November 2020 / Accepted: 9 December 2020
© The Author(s) 2021

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 Δku80* strain for efficient gene targeting. Dikaryons of *P. ostreatus Δmsh4 × Δmsh4* or *Δmer3 × Δmer3* 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 Δmsh4 × Δmsh4* dikaryons versus the *P. ostreatus msh4+ × Δmsh4* dikaryons, and 1/82.6 in the *P. ostreatus Δmer3 × Δmer3* dikaryons versus the *P. ostreatus mer3+ × Δmer3* dikaryons. To demonstrate the effects of *ku80* disruption, *P. ostreatus Δku80 × Δku80* 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 Δku80* 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 *Δku80* 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...
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/v) 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. 2016b). 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 μg and 1 μg, respectively) was introduced into protoplasts of *P. ostreatus* 20b (about 5 × 10⁷) to obtain the disruptant of *msh4*. A mixture of pBS-Δ*mer3* and *mer3*-disrupting cassette (about 2 μg and 1 μg, respectively) was also introduced into protoplasts of *P. ostreatus* 20b (about 5 × 10⁷) 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 (× 10^6) |
|-----------------|----------------------|----------------------------------|-------------------------------|
| PC9             | A2B1                 | Larraya et al. (1999)            |                               |
| PC15            | A1B2                 | Larraya et al. (1999)            |                               |
| #64             | A64B64               | Nakazawa et al. (2017a)          |                               |
| 20b             | A2B1 ku80: Cbx^R    | Salame et al. (2012)             |                               |
| 20b×PC15        | A2B1 A1B2            | This study                       |                               |
| 20b×#64         | A2B1 A64B64          | This study                       |                               |
| Δmsh4#1         | A2B1 ku80: Cbx^R msh4::hyg^R a msh4 disruptant derived from 20b | This study                       |                               |
| Δmer3#1         | A2B1 ku80: Cbx^R mer3::hyg^R /a mer3 disruptant derived from 20b | This study                       |                               |
| msh4^*×Δmsh4#1 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 880                           |
| msh4^*×Δmsh4#2 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 112                           |
| msh4^*×Δmsh4#3 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 370                           |
| Δmsh4^*×Δmsh4#1 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 0.1                           |
| Δmsh4^*×Δmsh4#2 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 132                           |
| Δmsh4^*×Δmsh4#3 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 0                             |
| Δmsh4^*×Δmsh4#4 | A2B1 A64B64 / a cross between Δmsh4#1 and the hyg^R F1 strain from Δmsh4#1 and #64 | This study                       | 0.9                           |
| Δmer3^*×Δmer3#1 | A2B1 A1B2 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and PC15 | This study                       | 188                           |
| Δmer3^*×Δmer3#2 | A2B1 A64B64 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and #64 | This study                       | 496                           |
| Δmer3^*×Δmer3#1 | A2B1 A1B2 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and PC15 | This study                       | 0                             |
| Δmer3^*×Δmer3#2 | A2B1 A64B64 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and #64 | This study                       | 5                             |
| Δmer3^*×Δmer3#3 | A2B1 A64B64 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and #64 | This study                       | 0.1                           |
| Δmer3^*×Δmer3#4 | A2B1 A64B64 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and #64 | This study                       | 7.2                           |
| Δmer3^*×Δmer3#5 | A2B1 A64B64 / a cross between Δmer3#1 and the hyg^R F1 strain from Δmer3#1 and #64 | This study                       | 8.4                           |
| m4#1            | A64B64 ku80: Cbx^R msh4:: hyg^R /a the F1 strain from Δmsh4#1 and #64 | This study                       |                               |

*a The carboxin-resistance marker

*b hyg^S and hyg^R indicate hygromycin-sensitive and -resistant, respectively
### Results

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

Approximately 10 hygromycin-resistant Δmsh4 or Δmer3 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 Δmsh4 transformants, disruption of Δmsh4 could be proven in one out of one transformant, and for the Δmer3 transformants, disruption of Δmer3 could be proven in three out of five transformants (Fig. 1c, d, e, and f).

**Table 2** The primers used in this study

| Primer | Sequence (5′ - 3′) |
|--------|-------------------|
| FY9    | TTAAGCATTACGACGCTACC |
| FY14   | AGACGTACCTGATAGCTTCGTC |
| FY15   | ACGCCCAATTCTTATGCGTTC |
| FY16   | ACTTACTCTTCTGAGCGCCGAC |
| FY17   | TACTCGTCAGTCGGTACTTC |
| FY18   | ACGCCCAATTCTTATGCGTTC |
| FY19   | ACGCCCAATTCTTATGCGTTC |
| FY20   | CCTGTGTAATTTGTTGGCCTCAGCTGTATATAAG |
| FY21   | ATAGTATTCGGAACACCCCGAGC |
| FY22   | TTAGTAATTAGGCGCATCTTCG |
| FY23   | AGTTCAGGATATCCCGCATG |
| FY24   | TCTGTGTTGCAACACATATTC |
| FY31   | TGCTCGTCCTGAGCTACGGTC |
| FY32   | ACGCCCAATTCTTATGCGTTC |
| FY33   | CCTGTGTAATTTGTTGGCCTCAGCTGTATATAAG |
| FY34   | TGCAACCGGAGCGGCTACGGTC |
| M13R   | ACAATTTACACAGGAAACAGCTATGACC |
| TN48   | TGCTCGTCCTGAGCTACGGTC |
| TN378  | TGCAATCTCAGCGCAGGATTAAGAC |
| TN400  | TCCAGTCAGGCAGGCAAAAACCCAGATACGCC |

Fig. 1 Targeted disruption of *msh4* or *mer3*. a A diagram illustrating the procedure for the construction of pBS-Δmsh4. 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-Δmer3. The red box indicates the 5′-side ORF of the *mer3* gene (1539 bp), and green the 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*. The arrows indicate the primers used for the PCR experiments in e. d A diagram of the genomic locus of *mer3*. The 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* Δ*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 PCR experiments confirming the *mer3* disruption. Lane 1, negative control (genomic DNA was not added); Lane 2, *P. ostreatus* 20b; Lane 3, *P. ostreatus* Δ*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 (×10^6) a | No. of examined strains |
|-------------------------|--------------------------------|------------------------|
| 20b × PC15              | 844 ± 545                      | 5                      |
| 20b × #64               | 824 ± 394                      | 5                      |
| msh4 Δ msh4             | 454 ± 319                      | 3 b, c                 |
| Δmsh4 × Δmsh4           | 33.3 ± 57.0                    | 4 b, d                 |
| mer3 Δ mer3             | 342 ± 154                      | 2 b, e                 |
| Δmer3 × Δmer3           | 4.14 ± 3.51                    | 5 b, f                 |

a Values indicate means ± standard deviations
b The number of basidiospores was measured once per each strain
c Three dikaryotic strains: msh4 × msh4#1–#3 (Table 1)
d Four dikaryotic strains: Δmsh4+ × Δmsh4#1–#4 (Table 1)
e Two dikaryotic strains: mer3 × Δmer3#1, #2 (Table 1)
f Five dikaryotic strains: Δmer3 × Δmer3#1–#5 (Table 1)

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

Dikaryotic strains P. ostreatus Δmsh4 × Δmsh4, msh4 Δ msh4, Δmer3 × Δmer3, and mer3 Δ mer3 were obtained to measure the number of basidiospores. To create the dikaryons, P. ostreatus Δmsh4#1 was crossed with P. ostreatus #64, and P. ostreatus Δmer3#1 was crossed with P. ostreatus PC15 or P. ostreatus #64 (Table 1). F1 strains were obtained from each cross (P. ostreatus Δmsh4#1 × #64, Δmer3#1 × PC15, and Δmer3#1 × #64). F1 progeny exhibiting resistance to hygromycin B (hygR) are considered as Δmsh4 or Δmer3, and those that were sensitive to hygromycin B (hygS) as msh4+ or mer3+. The F1 strains were mated with P. ostreatus Δmsh4#1 or Δmer3#1 to generate dikaryons (Table 1; three P. ostreatus msh4+ × Δmsh4, four Δmsh4 × Δmsh4, two mer3+ × Δmer3, and five Δmer3 × Δmer3). 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 Δmsh4 × Δmsh4 dikaryons versus the P. ostreatus Δmer3 × Δmer3 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+ × Δmer3 and Δmer3 × Δmer3 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 Δmsh4 × Δmsh4#2) was 1.32 × 10^8 and significantly differed from the other P. ostreatus Δmsh4 × Δmsh4 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 Δmsh4#1 × PC15 and P. ostreatus Δmsh4#1 × #64. F1 progeny exhibiting resistance to carboxin (cbxR) are considered Δku80, and those sensitive to carboxin (cbxS) as ku80+. We attempted to obtain F1 progeny with A64B64, cbxR and hygS backgrounds from P. ostreatus Δmsh4#1 × #64; however, we could not. Therefore, a monokaryon with A64B64, cbxR and

Table 4 Effects of ku80 disruption on basidiospore production

| Strain                  | No. of basidiospores (×10^6) a | No. of examined strains |
|-------------------------|--------------------------------|------------------------|
| 20b × PC15              | 759 ± 613                      | 10                     |
| 20b × #64               | 803 ± 979                      | 9                      |
| Δku80Δmsh4 × ku80Δmsh4+ | 718 ± 479                      | 20 b, c                |
| Δku80Δmsh4 × Δku80Δmsh4+| 1200 ± 681                     | 5 b, d                 |

a Values indicate means ± standard deviations
b The number of basidiospores was measured once per each strain
c 20 dikaryotic strains: Δku80Δmsh4 × ku80Δmsh4+ #1–#20
d Five dikaryotic strains: Δku80Δmsh4 × Δku80Δmsh4+ #1–#5
hygR 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 cbxS/hygS and five cbxR/hygS F1 strains from *P. ostreatus* Δmsh4#1×PC15 (the former ones were designated as *P. ostreatus* Δku80Δmsh4#1–#20 and the latter were designated *P. ostreatus* Δku80Δmsh4×Δku80msh4#1–#5). As shown in Table 4, there was no significant difference in the number of basidiospores between *P. ostreatus* ku80+×Δku80 and Δku80×Δku80 (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×PC15, msh4*+×Δmsh4*, and mer3*+×Δmer3* strains, but not in those of *P. ostreatus* Δmsh4×Δmsh4 and Δmer3×Δmer3 ones. In the *P. ostreatus* Δmsh4×Δmsh4 and Δmer3×Δmer3 strains, basidia with one large nucleus at prophase I (Fig. 2b: 3 and 6), which were not observed in the *P. ostreatus* 20b×PC15, msh4*+×Δmsh4*, and mer3*+×Δmer3* 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.
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 Δmsh4Δmsh4 dikaryons versus the P. ostreatus msh4Δmsh4 dikaryons, and 1/82.6 in the P. ostreatus Δmer3Δmer3 dikaryons versus the P. ostreatus mer3Δmer3 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 Δmer3Δmer3 strains whereas the numbers were reduced but varied widely in the P. ostreatus Δmsh4Δmsh4 strains possibly depending on their genetic backgrounds.

Basidia harboring two nuclei were not observed in the P. ostreatus Δmsh4Δmsh4 and Δmer3Δmer3 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 Δmsh4Δmsh4 and Δmer3Δmer3 strains (Fig. 2b: 4 and 7). In the case of C. cinereal, where meiotic progression is synchronized (Kies 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 Δmsh4Δmsh4 and Δmer3Δmer3 strains, apoptosis of nuclei may occur in the meiosis arrested basidia in this fungus.

A few basidiospores were produced in some P. ostreatus Δmsh4Δmsh4 or Δmer3Δmer3 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 premeiotic 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 ku80Δku80 and Δku80Δku80 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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11557-020-01661-w.

Acknowledgments We thank Prof. Yitzhak Hadar and Dr. Tomer M, Salame (Hebrew University of Jerusalem) for providing P. ostreatus strain 20b.

Authors’ contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Fuga Yamasaki, Takehito Nakazawa and Yoichi Honda. The first draft of the manuscript was written by Fuga Yamasaki and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This study was supported in part by JSPS KAKENHI (Grant No. 18KK0178 to Y.H.).

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.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.
References

Baars JJP, Sonnenberg ASM, Mikosch TSP, Van Grienven LJLD (2000) Development of a sporeless strain of oyster mushroom Pleurotus ostreatus. In: Grienven V (ed) Science and cultivation of edible fungi. Balkema, Rotterdam, pp 317–322

de Jong IF, Oom RA, de Bekker C, Wöstven HAB, Lugones LG (2010) Inactivation of kus80 in the mushroom-forming fungus Schizophyllum commune increases the relative incidence of homologous recombination. FEMS Microbiol Lett 310:91–95. https://doi.org/10.1111/j.1574-696x.2010.02052.x

Inada K, Morimoto Y, Arima T, Murata Y, Kamada T (2011) Homologous expression of recombinant manganese peroxidase genes in ligninolytic fungus Pleurotus ostreatus. Appl Microbiol Biotechnol 65:287–294. https://doi.org/10.1007/s002530000540

Knabe N, Jung EM, Freihorst D, Hennicke F, Horton JS, Kothe E (2013) A central role for Ras1 in the morphogenesis of the Basidiomycete Schizophyllum commune. Eukaryot Cell 12:941–952. https://doi.org/10.1128/EC.00355-12

Kück U, Hoff B (2010) New tools for the genetic manipulation of filamentous fungi. Appl Microbiol Biotechnol 86:51–62. https://doi.org/10.1007/s00253-009-0421-7

Kües U (2000) Life history and developmental processes in the Basidiomycete Coprinus cinereus. Microbiol Mol Biol Rev 64:316–353. https://doi.org/10.1128/MMBR.64.2.316-353.2000

Laraya LM, Perez G, Pe nas MM, Baars JJP, Mikosch TSP, Psarobaro AG, Ramirez L (1999) Molecular karyotype of the white rot fungus Pleurotus ostreatus. Appl Environ Microbiol 65:3413–3417

Levy M, Erental A, Yarden O (2008) Efficient gene replacement and direct hyphal transformation in Sclerotinia sclerotiorum. Mol Plant Pathol 9:719–725. https://doi.org/10.1111/j.1364-3703.2008.00483.x

Liebe B, Petukhova G, Barchi M, Bellani M, Braselmann H, Nakano T, Pandita TK, Jasim M, Fornace A, Meistrich ML, Baarends WM, Schimenti J, de Lange T, Keeney S, Camerini-Otero RD, Scherthan H (2006) Mutations that affect meiosis in male mice influence the dynamics of the mid-preleptotene and bouquet stages. Exp Cell Res 312:3768–3781. https://doi.org/10.1016/j.yexcr.2006.07.019

Nakagawa T, Ogawa H (1999) The ligninolytic system through an efficient forward genetics in the white-rot agaricomycete Pleurotus ostreatus. Environ Microbiol 19:261–272. https://doi.org/10.1111/j.1462-2920.13595

Nakazawa T, Iznou A, Horii M, Kodera R, Nishimura H, Hirayama Y, Tsunematsu Y, Miyazaki Y, Awano T, Muraguchi H, Watanabe K, Sakamoto M, Takabe K, Watanabe T, Isagi Y, Honda Y (2017b) Effects of pex1 disruption on wood lignin biodegradation, fruiting development and the utilization of carbon sources in the white-rot Agaricomycete Pleurotus ostreatus and non-wood decaying Coprinopsis cinerea. Fungal Genet Biol 109:7–15. https://doi.org/10.1016/j.fgb.2017.10.002

Nakazawa T, Morimoto R, Wu HL, Kodera R, Sakamoto M, Honda Y (2019) Dominant effects of gapl mutations on the ligninolytic activity of the white-rot fungus Pleurotus ostreatus. Fungal Biol 123:209–217. https://doi.org/10.1016/j.funbio.2018.12.007

Namekawa S, Ichijima Y, Hamada F, Kasai N, Ibakata K, Nara T, Teraoka H, Sugawara H, Yanai T, Daikuhara Y, Sakaguchi K (2003) DNA ligase IV from a basidiomycete, Coprinus cinereus, and its expression during meiosis. Microbiol 149:2119–2128. https://doi.org/10.1099/mic.0.26311-0

Ninomiya Y, Suzuki I, Ishii C, Honda Y (2004) Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining. PNAS 101:12248–12253. https://doi.org/10.1073/pnas.0407377101

Okuda Y, Murakami S, Honda Y, Matsumoto T (2013) An MSH4 homolog, sptpl, from Pleurotus pulmonarius is a “silver bullet” for resolving problems caused by spores in cultivated mushrooms. Appl Environ Microbiol 79:4520–4527. https://doi.org/10.1128/AEM.00561-13

Rao PS, Niederpruem DJ (1969) Carbohydrate metabolism during morphogenesis of Coprinus lagopus (sensu Buller). J Bacteriol 100:1222–1228. https://doi.org/10.1128/JB.100.3.1222-1228.1969

Ruiz-Diez B (2002) Strategies for the transformation of filamentous fungi. J Appl Microbiol 92:189–195. https://doi.org/10.1046/j.1365-2672.2002.01516.x

Salame TM, Knop D, Tal D, Levinson D, Yarden O, Hadar Y (2012) Predominance of a versatile-peroxidase-encoding gene, mnp4, as demonstrated by gene replacement via a gene targeting system for Pleurotus ostreatus. Appl Environ Microbiol 78:5341–5352. https://doi.org/10.1128/AEM.01234-12

Snowden T, Acharya S, Butz C, Berardini M, Fishel R (2004) hMSH4-hMSH5 recognizes Holliday junctions and forms a meiosis-specific sliding clamp that embraces homologous chromosomes. Mol Cell 15:437–451. https://doi.org/10.1016/j.molcel.2004.06.040

Sugawara H, Iwabata K, Koshikyama A, Yanai T, Daikuhara Y, Namekawa S, Hamada F, Sakaguchi K (2003) Coprinus cinereus Mre3 is required for synaptonemal complex formation during meiosis. Chromosoma 118:127–139. https://doi.org/10.1007/s00442-008-0815-1

Tsuikihara T, Honda Y, Sakai R, Watanabe T (2006) Excessive overproduction of recombinant versatile peroxidase MnP2 by genetically modified white rot fungus, Pleurotus ostreatus. J Biotechnol 126:431–439. https://doi.org/10.1016/j.jbiotec.2006.05.013

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.