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Pleurotus ostreatus mek1 is essential for meiosis and basidiospore production

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ABSTRACT

Breeding strains without basidiospores is important for the mushroom industry. However, target genes for sporeless breeding remain limited. To identify a new gene essential for basidiospore production in *Pleurotus ostreatus*, the RNA sequencing data obtained in our previous study were re-analyzed. Among the 36 *P. ostreatus* genes that are exclusively expressed in the gills where basidiospores are formed and produced, candidate genes were narrowed down using transcriptome data during the fruiting stage of *Coprinopsis cinerea*, in which the meiotic steps progress synchronously. Three *C. cinerea* genes homologous to the gill-specific *P. ostreatus* genes were upregulated during meiosis. One of these three genes encodes a protein homologous to *Saccharomyces cerevisiae* Mek1p, a protein kinase important in the meiotic recombination checkpoint. Plasmids containing expression cassettes for hygromycin B-resistance screening, Cas9, and single-guide RNA targeting *mek1* were introduced into the protoplasts of the dikaryotic *P. ostreatus* strain PC9×#64, which showed that the obtained dikaryotic gene disruptant produced no basidiospores. Microscopic analysis suggests that meiosis is suspended during telophase I. These results suggested that *P. ostreatus* Mek1 is essential for meiosis II progression and basidiospore production. In addition, disturbed orientation and loss of negative gravitropism during fruiting were observed.

1. Introduction

Sexual reproduction in *Agaricomycetes* is characterized by the formation of relatively large fruiting bodies, generally referred to as mushrooms, that produce and disperse basidiospores (Kües and Navarro-González, 2015). Mushrooms are often represented as symbols of fungal multicellular morphogenesis, and many studies have been conducted to elucidate the mechanisms underlying fruiting development in *Agaricomycetes*, particularly using *Coprinopsis cinerea* and *Schizophyllum commune* as models (Kamada, 2002; Palmer and Horton, 2006).

Many agaricomycetes, including *Agaricus bisporus* (button mushroom), *P. ostreatus* (oyster mushroom), *Lentinula edodes* (shiitake mushroom), and *Ganoderma lucidum* (lingzhi or reishi), are cultivated worldwide. Extensive efforts are ongoing to generate strains with useful cultivation traits, such as high productivity and quality, for the mushroom industry (Eger et al., 1976; Barh et al., 2019). Breeding "sporeless" strains with no basidiospore production is also important as they may avoid allergic reactions in employees in mushroom cultivation facilities and facility disruption (Baars et al., 2000) and preserve biodiversity at the genetic level (Okuda, 2022). Therefore, substantial efforts have been devoted to the generation of sporeless-cultivated strains using classical methods such as crossing and random mutagenesis (Eger et al., 1976; Ohira, 1979; Hasebe et al., 1991; Obatake et al., 2003; Pandey and Ravishankar, 2010; Yoneyama et al., 2020). Molecular genetic studies have also been conducted in *C. cinerea* (Cummings et al., 1999; Sugawara et al., 2009), *S. commune* (Lavrijssen et al., 2020), *Pleurotus pulmonarius* (Okuda et al., 2013), and *P. ostreatus* (Lavrijssen et al., 2020; Yamasaki et al., 2021, 2022) to identify genes whose inactivation impairs basidiospore production. These studies have focused on meiosis-related genes, like *mer3* and *msh4*, as their defects in meiosis can impair basidiospore production. Recently, we identified two genes, *pcl1* and *cro6c*, which are essential for basidiospore formation after meiotic division based on comparative transcriptomic analyses; genes exclusively expressed in the gills of *P. ostreatus* and upregulated in the caps of *C. cinerea* at/after the completion of meiosis II were selected (Kobukata et al., 2024). However, the molecular mechanism for meiotic division

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and sprore formation remains to be elucidated.

In this study, we re-analyzed comparative transcriptome data to identify a new gene that is essential for meiotic division and also a target for the breeding of sporeless strains, to extend knowledge on meiosis in *Agaricomycetes*.

2. Materials and methods

2.1. Strains, media, culture conditions, and genetic manipulation

The *P. ostreatus* strains used in this study are listed in Table 1. Routine cultures, fruiting, and hygromycin resistance transformations were performed as described by Kobukata et al. (2024), Nakazawa et al. (2016), and Salame et al. (2012), respectively.

2.2. Analysis of transcript accumulation by RNA sequencing (RNAseq) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Fruiting bodies from dikaryotic *P. ostreatus* strains N001 (PC9×PC15) and PC9×#64 with fully expanded pilei were harvested, followed by separation of stipes, gills, and pilei without gills, as described by Kobukata et al. (2024). Mycelial cells of PC9×#64 grown on the plastic-bottled sawdust medium used for fruiting were also harvested three days after the temperature downshift from 28°C to 18°C for fruiting (no visible structures such as primordia and hyphal knots were observed at this time). Total RNA extraction and qRT-PCR were performed as described by Kobukata et al. (2024) and Nakazawa et al. (2023a), respectively. The primer pairs used for qRT-PCR and their amplification efficiencies are listed in Table S1.

2.3. Rapid amplification of cDNA end (RACE)

We performed 5′- and 3′-RACE with total RNA isolated from gill using the SMARTer RACE 5′/3′ kit (Takara, Shiga, Japan). Primer pairs TN1621/UPM (Universal primer A mix attached in the kit) and TN1624/SMARTer_nested were used for the first and second PCR reaction for 3′-RACE; TN1623/UPM, TN1622/SMARTer_nested, and TKB10/SMARTer_nested were used for the first, second, and third PCR reaction for 5′-RACE (Table S2). The amplified fragments were separately cloned into pBluescript II SK + (Agilent Technologies, Santa Clara, CA, USA) and digested with EcoRV, followed by DNA sequencing using primers M13F or M13R (Table S2).

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

Strain	Genotype/description	Source
PC9	*A2B1*	Larraya et al. (1999)
#64	*A64B64*	Nakazawa et al. (2017)
PC9×#64	*A2B1 A64B64*/a cross between PC9 and #64	Kobukata et al. (2024)
20b	*A2B1 ku80*::*cbx*^{Ra}	Salame et al. (2012)
*mek1*g1#1–#4	*A2B1 A64B64 hyg*^{R}/four dikaryotic transformants obtained by introducing pCcPef3-126-*mek1*sg1	This study
*mek1*g2#1–#2	*A2B1 A64B64 hyg*^{R}/two dikaryotic transformants obtained by introducing pCcPef3-126-*mek1*sg2	This study
F_1#2	*A64B64 mek1*$^{-}$/the F_1 strain from *mek1*g2#2 and #64	This study
*pcl1*g1#1	*A2B1 A64B64 pcl*$^{-}$ *hyg*^{Ra}/a dikaryotic *pcl1* disruptant	Kobukata et al. (2024)
*cro6c*g1#3	*A2B1 A64B64 cro6c*$^{-}$ *hyg*^{Ra}/a dikaryotic *cro6c* disruptant	Kobukata et al. (2024)

[a]*cbx*^{R}and *hyg*^{R} indicate carboxin and hygromycin resistance genes from pTM1 (Honda et al., 2000) and pPHT1 (Cummings et al., 1999), respectively.

2.4. Design of mek1-targeting sequences in single-guide RNA (sgRNA)

Two sgRNA sequences, *mek1*sg1 and *mek1*sg2, targeting nucleotide positions 33–52 and 1445–1464 (34 bp upstream from the stop codon), respectively, from the start codon determined by RACE experiments, were designed in accordance with the method described by Boontawon et al. (2021a). It was confirmed that each target sequence in each designed sgRNA was available in *P. ostreatus* strain #64 because there were no differences in these 20 bp sequences between PC9 and #64 when analyzing whole-genome resequencing data from #64 (Yamasaki et al., 2022).

2.5. Plasmid construction

The pCcpef3-126-based plasmids for sgRNA expression, along with recombinant Cas9 harboring triple nuclear localization signals and a hemagglutinin tag at the C-terminus (Boontawon et al., 2021a, 2021b), were constructed as described by Sugano et al. (2017). In brief, double-stranded DNAs prepared by annealing two synthesized DNA oligos, FY67/FY68 (*mek1*sg1) and FY69/FY70 (*mek1*sg2) (Table S2), were separately inserted into the BsaI site of linearized pCcpef3-126. The resulting plasmids were designated as pCcPef3-126-*mek1*sg1 and pCcPef3-126-*mek1*sg2, respectively.

2.6. Rapid genomic PCR

Genomic DNA was extracted using the method described by Nakazawa et al. (2016), and genomic PCR experiments were performed using KOD FX Neo (TOYOBO, Tokyo, Japan).

2.7. Basidiospore measurement

The number of basidiospores that fell from the fruiting body onto the Petri dish within 3 days was counted using a hemocytometer, as described by Kobukata et al. (2024).

2.8. Observation using scanning electron microscopy (SEM)

Observations of basidia and basidiospores in the gill tissues were carried out using SEM JSM-7900F (JEOL, Tokyo, Japan), as described by Han et al. (2023a, 2023b).

2.9. Fluorescence staining of nuclei in basidia

Gills (approximately 2 mm × 2 mm × 2 mm) were harvested from fruiting bodies with fully expanded pilei (Kobukata et al., 2024), followed by staining with Hoechst 33342 (Bio-Rad, Hercules, CA, USA) in ProLong Glass Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature (20–28°C). Observations were performed under an AxioScope A1 fluorescence microscope (ZEISS, Oberkochen, Germany) equipped with a filter set of 91 HE.

3. Results

3.1. Identification of P. ostreatus mek1 gene as a candidate

Kobukata et al. (2024) listed 36 *P. ostreatus* genes exclusively expressed in the gills of fruiting bodies from N001 (PC9×PC15) at the transcription level based on transcriptome analysis, followed by searching *C. cinerea* genes homologous to the 36 *P. ostreatus* genes in the JGI genome database (https://mycocosm.jgi.doe.gov/Copci_AmutBmut1/Copci_AmutBmut1.home.html) (Table S3). Our previous study focused on genes upregulated 12 h after karyogramy, K + 12 in Burns et al. (2010) or 36 h in Muraguchi et al. (2015) in *C. cinerea*, to identify

genes essential for basidiospore formation after meiotic division in *P. ostreatus* (Kobukata et al., 2024). In this study, we reanalyzed comparative transcriptome data to identify genes essential for meiosis progression and basidiospore production.

C. cinerea genes that play role(s) in specific meiotic process(es) are highly upregulated and sometimes predominantly expressed during the corresponding stages from K + 0 to K + 12 at the transcriptional level (Sugawara et al., 2009; Burns et al., 2010); therefore, *C. cinerea* genes homologous to the gill-specific *P. ostreatus* genes that were upregulated or predominant during K + 0–12 were selected using transcriptome data (Muraguchi et al., 2015). Three genes, CC1G_13697, CC1G_12220, and CC1G_01117 were upregulated at K + 0 and K + 6 (Fig. S1). Protein IDs corresponding to *P. ostreatus* genes homologous to the three *C. cinerea* genes are 96060, 59729, and 82484. Blastp search suggested that 96060 encodes a putative protein kinase that does not exhibit homology to any known proteins; 59729 is an agaricomycetes-specific protein without any known motifs and domains; and 82484 is *mer3*.

Silencing and disrupting *mer3* resulted in the suspension of meiosis in prophase I, causing reduced basidiospore production in *C. cinerea* (Sugawara et al., 2009) and *P. ostreatus* (Yamasaki et al., 2021, 2022). Considering that protein phosphorylation participates in meiotic checkpoints (Subramanian and Hochwagen, 2014), we selected the putative protein kinase-encoding gene, 96060, as a target.

To determine the amino acid sequence of a protein encoded by 96060, 5′- and 3′-RACE were performed. Four cloned 5′-RACE fragments were sequenced, revealing that the 5′ end of each fragment is 29, 25, 25, and 24 bp upstream of the start codon. Four cloned 3′-RACE fragments were also sequenced. Although the precise 3′ end could not be determined as the waveforms around poly dT were distorted when sequenced from both 5′ and 3′ directions, the 3′ end is around position 182760 of Scaffold 4 in the genome database of *P. ostreatus* PC9 by the Joint Genome Institute [JGI (https://mycocosm.jgi.doe.gov/PleosPC9_1/PleosPC9_1.home.html]. This is approximately 800 bp downstream of that predicted by JGI. Based on these results together with sequencing of cDNA, the gene corresponding to protein ID 96060 was found to be interrupted by 13 introns (Fig. 1A) and to encode a 459-amino acid protein with forkhead-associated (FHA) and protein kinase domains (Fig. 1B; DDBJ accession No. LC856601).

Considering that the determined amino acid sequence encoded by 96060 was different from that predicted by JGI, a BLASTP search was carried out again to examine if the determined amino acid sequence exhibited similarity to known protein(s). The results showed that *Saccharomyces cerevisiae* Mek1p exhibited high similarity [*E*-value, 2e-44; amino-acid identity, 30.7 %; Query cover, 91 %]. This serine/threonine protein kinase functions at the meiotic recombination checkpoint (Rockmill and Roeder, 1991; Niu et al., 2007). Reciprocal BLASTP was performed on the amino acid sequence of *Sc*Mek1p to confirm its correspondence with 96060. All other proteins predicted in the JGI *P. ostreatus* database exhibited a lower similarity than 96060. This result suggests that the gene corresponding to protein ID 96060 encodes a protein homologous to *Sc*Mek1p; therefore, it was designated as *P. ostreatus mek1*.

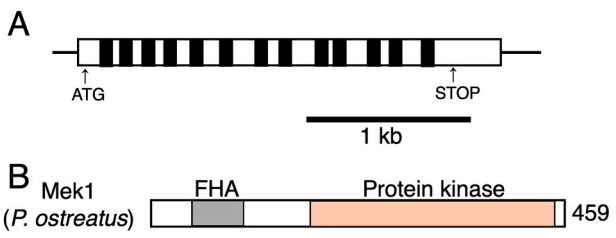

Fig. 1. *Pleurotus ostreatus mek1* gene determined in this study. (A) Structure of the *mek1* gene. Open boxes and solid boxes indicate exons and introns, respectively. (B) Predicted structural feature of the Mek1 protein.

3.2. Fruiting bodies of the dikaryotic *P. ostreatus mek1* disruptants produced no basidiospores

In this study, the dikaryotic strain PC9×#64 was used as the recipient strain for *mek1* disruption in both nuclei using plasmid-based CRISPR/Cas9, as described by Yamasaki et al. (2022), while that used for comparative RNA-seq analysis was strain N001 (Kobukata et al., 2024). Therefore, prior to *mek1* disruption, qRT-PCR was performed to confirm whether *mek1* transcript accumulated abundantly in the gills of fruiting bodies of PC9×#64. The most abundant accumulation of transcripts was observed in the gill tissues among the three parts of the fruiting bodies from PC9×#64 (Fig. 2). The relative transcript abundance in gill tissues was100-fold higher than that in mycelial cells, with a similar *mek1* expression pattern between N001 and PC9×#64.

Subsequently, two different plasmids, pCcPef3-126-*mek1*sg1 and pCcPef3-126-*mek1*sg2, were independently introduced into protoplasts from PC9×#64 to generate dikaryotic gene disruptnats in a single transformation experiment. Four and two hygromycin-resistant transformants were obtained using the respective plasmids. All six transformants showed the presence of clamp cells, indicating that they were dikaryotic. These strains were designated as *mek1*g1#1–#4 and *mek1*g2#1–#2 (Table 1).

The number of basidiospores produced by or dispersed from the fruiting bodies of each dikaryotic transformant was determined to examine whether the introduction of each plasmid conferred sporeless phenotypes on PC9×#64. PC9×#64 produced approximately 1400 × 10^6 basidiospores, whereas one transformant, *mek1*g2#2, produced no basidiospores in three biological replicates (Fig. 3A). This result suggests that strain *mek1*g2#2 is sporeless.

Genomic PCR experiments were subsequently performed to examine whether the mutations in *mek1* were introduced into the nuclei of each transformant (Fig. 3B). The analysis was first performed on the four *mek1*g1 and two *mek1*g2 strains using the primer pairs TKB29/TKB30 and TKB1/TKB2 (Table S2), respectively, as a positive control, amplifying the open reading frame (ORF) of *mek1* outside of the target site for *mek1*sg1 (552 bp; at nucleotide positions 1120–1671 from the start

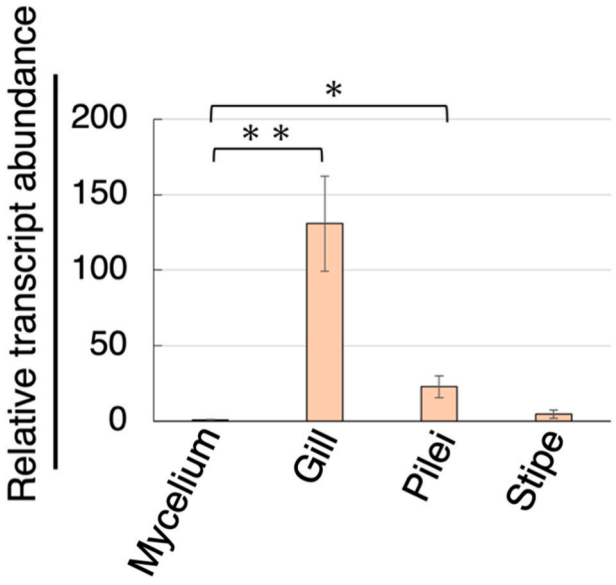

Fig. 2. Relative transcript accumulation of *mek1* in mycelial cells 3 days following temperature downshift for fruiting induction (mycelium) on bottled sawdust medium and each part of fruiting bodies (gill, pilei, and stipe; Kobukata et al., 2024) from PC9×#64 with fully expanded pilei. Graphs and bars indicate means and standard deviations, respectively (n = 3). Statistical significance tests were performed using a two-tailed equal variance t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

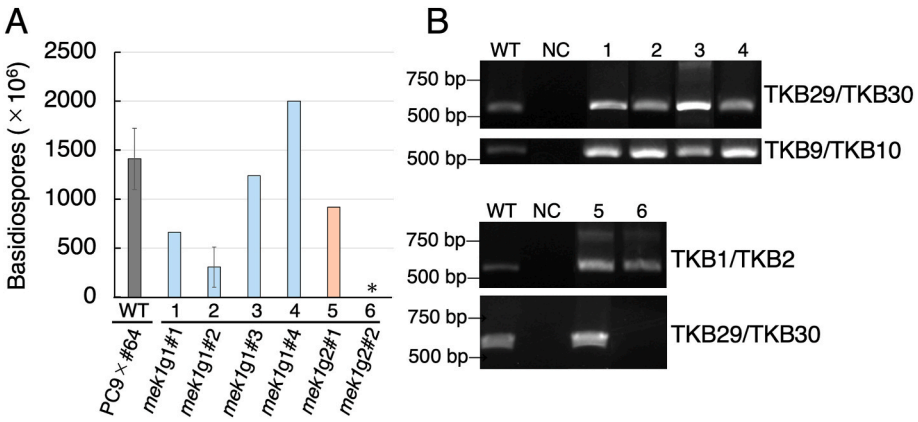

Fig. 3. Basidiospore production and gene disruption in the obtained transformants. (A) Number of basidiospores produced by/dispersed from each transformant (lanes 1–6) along with the parental control, PC9×#64 (wild type, WT). Asterisks indicate strains with no basidiospore production (n = 3). The graphs demonstrate the means (n = 1–3), and the error bars indicate the standard deviations (n = 3 for WT, 2, and 6; n = 1 for 1 and 3–5). (B) Genomic PCR experiments examining *mek1* disruption with the respective primer pairs listed in Table S2.

codon) and the latter ORF (562 bp; nucleotide positions 75–637) of the gene corresponding to Protein ID 48224 (Kobukata et al., 2024). A genomic fragment of the expected size was amplified from all six strains (Fig. 2B).

Next, genomic PCR was performed with TKB9/TKB10 (on *mek1*g1#1–#4) or TKB29/TKB30 (on *mek1*g2#1–#2) to examine *merk1* disruption, which, respectively, amplified the ORF of *mek1* containing the target site for *mek1*sg1 (538 bp; at nucleotide positions −180 to 358 from the start codon) and *mek1*sg2 (552 bp; nucleotide positions 1120–1671). Lack of amplification has been frequently reported in *P. ostreatus* mutants generated using pCcPef3-126-based CRISPR/Cas9. In most cases, this was due to the longer extension time required by the mutants than that of the fragment amplified from the parental control strain (Boontawon et al., 2021a, 2021b; Yamasaki et al., 2022; Nakazawa et al., 2023b). Amplification of an approximately 500–600 bp genomic fragment was observed in all five strains that produced basidiospores, as well as in the parental control strain PC9×#64 (Fig. 3B). Only *mek1*g2#2, with no basidiospore production, showed no fragment amplification (lane 6, lower photo), whereas the positive control was amplified from this strain (lane 6, upper photo). Although small mutations, such as 1–10 bp insertions, deletions, and substitutions, may have been introduced in some of the other five strains, we did not analyze the nucleotide sequences of the fragments amplified with TKB9/TKB10 or TKB29/TKB30. These results suggest that at least *mek1*g2#2 may be a dikaryotic gene disruptant.

3.3. Genetic analysis supported that mek1 disruption is responsible for sporeless phenotype

Based on our results, the *mek1* disruption may be responsible for the sporeless phenotype of *mek1*g2#2. However, the possibility that the ectopic integration of the introduced plasmid(s) or off-target effects (s) could have caused the mutant phenotypes cannot be ruled out. Moreover, it was not revealed how *mek1* was disrupted in *mek1*g2#2. Therefore, genetic analyses were performed to confirm whether *mek1* is essential for basidiospore production.

Di–mon mating of *mek1*g2#2 (*A2B1* A64B64) with a wild-type monokaryon, PC9 (*A2B1*), resulted in a dikaryon that produced basidiospores [2240 ± 200 × 10^6 (n = 3)] similar to PC9×#64. This result suggests the recessive nature of the sporeless strain, *mek1*g2#2.

Based on this result, additional dikaryotic strains with *mek1*$^+$×*mek1*$^-$ and *mek1*$^-$×*mek1*$^-$ were generated by backcrossing, followed by observation of basidiospore production. To this end, 33 F_1 strains were isolated from a di–mon cross, *mek1*g2#2×PC9 or *mek1*g2#2×#64. They were mated with #64 or PC9 to screen for F_1 progeny with an *A2B1* or A64B64 background based on the observation of clamp cells. Five strains were identified and designated as F_1#1–#5 (Table 1). Genomic PCR experiments were performed to determine whether the five F_1 strains were *mek1*$^+$ or *mek1* disruptants. As the genomic fragment was not amplified from *mek1*g2#2 with TKB29/TKB30 (Fig. 3B), genomic PCR was used to determine whether the F_1 progeny were *mek1*$^+$ or disruptants. F_1#1, #3, #4, and #5 are *mek1*$^+$, whereas F_1#2 is a *mek1* disruptant (Fig. S2).

Next, F_1#4 was mated with *mek1*g2#2 or its parental control PC9×#64, followed by basidiospore count. A cross between *mek1*g2#2 and PC9×#64 (*mek1*$^+$×*mek1*$^-$) (n = 1) produced 1270 × 10^6 basidiospores, while no from a cross between *mek1*g2#2 and F_1#4 (*mek1*$^-$×*mek1*$^-$) in all triplicate biological replicates. This suggests that *mek1* disruption is responsible for the sporeless phenotype of *mek1*g2#2.

3.4. Most basidia observed in the mek1 disruptant contained two nuclei

Basidia in the gill tissue were observed by SEM to investigate the stage at which basidiospore production was impaired in the *mek1* disruptant. Basidiospores and sterigma were observed in the dikaryotic parental control strain PC9×#64 but not in *mek1*g2#2 (Fig. 4A).

Next, the gills of these strains were stained with Hoechst 33342 to visualize the nuclei in the basidia (Fig. 4B). Similar to the results of Kobukata et al. (2024), basidia at various stages of meiotic division with single, double, or quadruple spherical blue fluorescence (chromosomes or nucleus/nuclei) were observed in the parental control PC9×#64. By contrast, almost all basidia harvested from *mek1*g2#2 showed double blue fluorescence (Fig. 4B), and no basidium with quadruple fluorescence was found. Basidia with two nuclei are generally observed in telophase I (Sugawara et al., 2009) and before karyogamy. Therefore, meiosis may have been suspended at either stage in the *P. ostreatus mek1* disruptant, resulting in a sporeless phenotype. *C. cinerea mek1*, which corresponded to CC1G_13697, was highly upregulated at K + 6 and K + 12 (Fig. S1) around telophase I (K + 9; Sugawara et al., 2009). Given that the function of Mek1 is conserved between *P. ostreatus* and *C. cinerea*, meiosis is more likely to be suspended at telophase I in the *mek1* disruptant, and Mek1 is essential for the progression to meiosis II.

3.5. Disturbed orientation and loss of negative gravitropism of fruiting bodies were observed in the mek1 disruptant as well as in pcl1 or cro6c ones

The disturbed orientation was related to the absence of basidiospores in *Pleurotus eryngii* (Obatake et al., 2003) and *P. ostreatus* (Lavrijssen et al., 2020; Kobukata et al., 2024). To examine whether the *mek1*

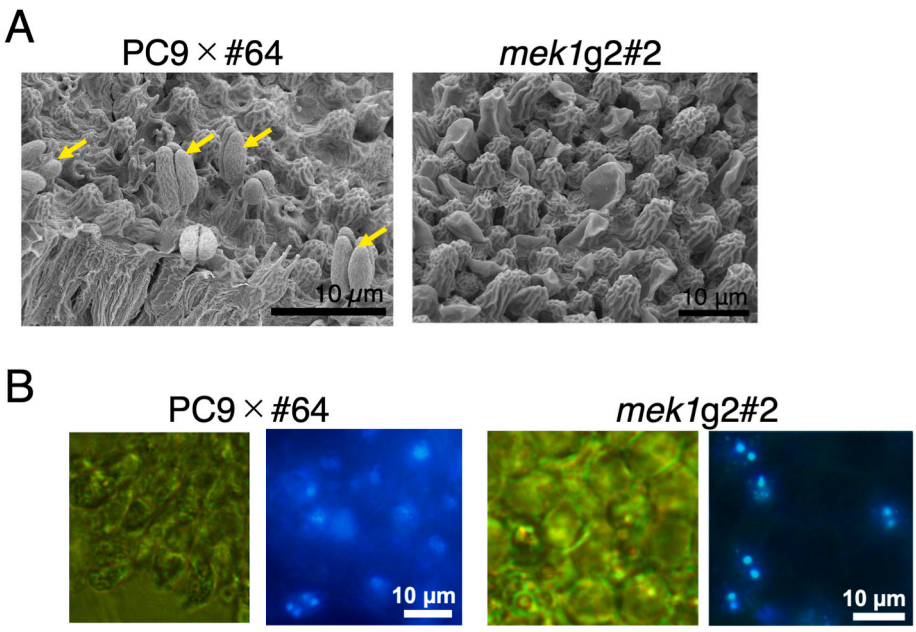

Fig. 4. Microscopic observation of basidial cells of PC9×#64 and *mek1*g2#2. (A) Observation of the surface of basidial cells in the indicated strains by scanning electron microscopy. Yellow arrow indicates basidiospores. Black scale bars = 10 μm. (B) Fluorescence microscopy of basidial cells stained with Hoechst 33342. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

disruptant also exhibited this phenotype, fruiting of *mek1*g2#2 was observed. Similar to *pcl1* or *cro6c* disruptants (Kobukata et al., 2024), the orientations of fruiting bodies from *mek1*g2#2 were disturbed compared to those of the parental control strain, PC9×#64 (Fig. S3).

A negative gravitropic orientation of fruiting bodies has been observed in many agaricomycetes, including *C. cinerea*, *Flammulina velutipes*, and *P. ostreatus* (Moore, 1991; Moore et al., 1996; Miyazaki et al., 2010). To examine the possibility that the observed disturbed orientation is related to gravitropism, we changed direction of the cultivation bottle immediately after small fruiting bodies formed such as those indicated in "Before" in Fig. 5. As shown in "After" in Fig. 5, the fruiting bodies from PC9×#64 bent upward to regain the vertical orientation 2 days after the bottles were laid down, while those from *mek1*g2#2, *pcl1*g1#1, and *cro6c*g1#3 bent to random directions as shown in Fig. S3. This suggests that negative gravitropism may have been lost in all three *P. ostreatus* strains.

4. Discussion

In this study, we identified the *mek1* genes essential for meiosis and basidiospore production in *P. ostreatus*, demonstrating an efficient and useful strategy for reverse genetics utilizing multiple transcriptome data. Our previous studies also identified genes, *pcl1* and *cro6c*, essential for basidiospore formation after meiotic division (Kobukata et al., 2024), while deletants and disruptants of the meiotic-related *mer3* or *msh4* do not lose the ability of *P. ostreatus* to produce basidiospores completely (Yamasaki et al., 2021, 2022). The present study suggested that a meiotic-related gene, *mek1*, may function separately from *mer3* and *msh4* and is a good candidate to be mutated for a completely sporeless breeding. It may serve a new target to investigate the fundamental mechanism in sexual development in *Agaricomycetes*.

In *S. cerevisiae*, Mek1p kinase activity modulates the functions of several proteins such as the recombinase Dcm1p to promote meiotic recombination initiated by double-strand break formation by Spo11p (Hollingsworth and Gaglione, 2019). In *C. cinerea*, the number of basidiospores produced by *lim15* (homologous to *S. cerevisiae DCM1*) knockdown strains (Namekawa et al., 2005) as well as by the *spo11* disruptant isolated by restriction enzyme-mediated mutagenesis (Celerin et al., 2000) also greatly reduced owing to the suspension of meiosis during meiosis I. However, in this study, we showed that the *P. ostreatus mek1* disruptant seemed to complete meiosis I (Fig. 4B). This inconsistency suggests that meiosis may not be suspended at the step involving Dcm1/Lim15 participation in the *P. ostreatus mek1* disruptant, which may be because Mek1p also participates in the checkpoint for

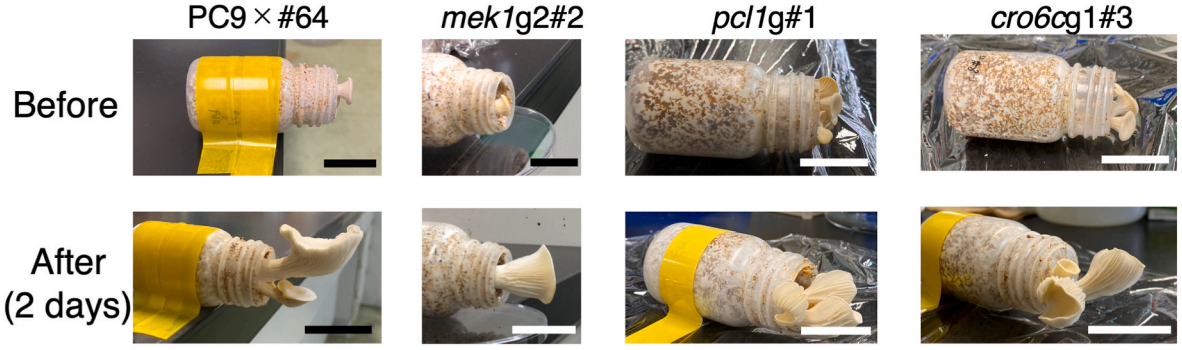

Fig. 5. Negative gravitropism of *mek1*g2#2 as well as the sporeless strains isolated by Kobukata et al. (2024). Upper images (before) show fruiting bodies just when they were turned sideways. Lower ones (after) those 2 days after turning sideways. Scale bars = 4 cm.

crossover formation after the step involving Dmc1p in *S. cerevisiae* (Hollingsworth and Gaglione, 2019), We also observed the different effects of *mek1* disruption on sexual spore production in *P. ostreatus* and *S. cerevisiae*. In *S. cerevisiae*, reduced viability of ascospores, but not a sporeless phenotype, was observed in *mek1* deletants (Rockmill and Roeder, 1991), whereas this was not observed in the *P. ostreatus mek1* disruptant. This difference may be due to the diverse mechanisms underlying the meiotic checkpoints in *P. ostreatus* and *S. cerevisiae*, even though the core meiotic expression program is conserved between the agaricomycete *C. cinerea* and ascomycetous yeasts (Burns et al., 2010).

In addition, the *P. ostreatus mek1* disruptant, as well as *pcl1* or *cro6c* ones, exhibited disturbed orientation and loss of negative gravitropism of fruiting bodies (Fig. 5 and S3; Kobukata et al., 2024). Although it remains unclear whether the two deficiencies in sporulation are directly related, similar deformed fruiting bodies have been frequently observed in sporeless *P. ostreatus* strains (Baars et al., 2000). Considering the results obtained in this study and Kobukata et al. (2024), the production of normal basidiospores possibly results in the determination of fruiting orientation or negative gravitropism in *P. ostreatus*. However, these observations of sporeless and abnormal fruiting bodies may depend on the species, as negative gravitropism has been observed in some *C. cinerea* sporeless mutants (Muraguchi et al., 1999; Casselton and Riquelme, 2004). It would be interesting to investigate the mechanisms underlying the observed link between spore formation and loss of negative gravitropism in *P. ostreatus* mutant strains in future studies.

CRediT authorship contribution statement

Takehito Nakazawa: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Takeshi Kobukata:** Investigation, Formal analysis, Data curation. **Fuga Yamasaki:** Methodology, Investigation, Formal analysis, Data curation. **Junko Sugano:** Investigation. **Minji Oh:** Project administration. **Moriyuki Kawauchi:** Investigation. **Yoichi Honda:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2025.101562.

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