Introduction and utilization of a gene targeting system in a basidiomycete *Pleurotus ostreatus* using CRISPR/Cas9 genome editing technology

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BOONTAWON TATPONG

ABBREVATIONS

Cbx^{R}	gene for carboxin resistance
CRISPR/Cas9	clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9
DSB	double-strand DNA break
5-FC	5-fluorocytosine
fcy1	gene for cytosine deaminase
5-FOA	5-fluoroorotic acid
HDR	homology-directed repair
hph	gene for hygromycin phosphotransferase
NHEJ	non-homologous end joining
NLS	nuclear localization sequences
pyrG	gene for orotidine 5'-phosphate decarboxylase
sgRNA	single guide RNA
WT	wild-type
YMG	Yeast and malt extract with glucose
YMGUU	YMG supplemented with 0.18 mM uracil and 20 mM uridine

CONTENTS

INTRODUCTION

Currently, the world's population has been increasing. In the 20th century, the total number of the world citizens stood at 1.6 billion and then ended at 6 billion people. In addition, the global population has been anticipated to reach 10 billion in 2050 (FAO, 2017). This may lead to three main consequences: an increasing of global food requirement, an indispensable medical need, and a severe pollution in the environment. Thus, the solutions to these expanding situations are required.

Fungi in a class of *Agaricomycetes*, which includes around 21,000 species, belong to the division of Basidiomycota (Kirk et al., 2008). Some fungi in this class produce large reproductive structures called mushrooms, which have been utilised for human consumption and/or medicinal purposes for many centuries. Important edible mushrooms in a class of *Agaricomycetes* such as *Agaricus bisporus* (champignon or button mushroom) and *Pleurotus ostreatus* (oyster mushroom). In the aspect of medicinal purposes, *Ganoderma lucidum* (lingzhi or reishi), *Lentinula edodes* (shiitake), and *Inonotus obliquus* (chaga) suggested to stimulate immune system and cure some cancer (Won et al., 2011; Guggenheim et al., 2014; Nagashima et al., 2017; Sohretoglu and Huang, 2018). Some agaricomycetes such as *Boletus edulis* (porcini) and *Cantharellus cibarius* (chanterelle) are mycorrhiza fungi, which is a type of plant-fungal symbiosis. Maintaining this relationship, which plays a key role in soil carbon storage, could be one of the important factors to slow climate change (Monz et al., 1994; Clemmensen et al., 2013). Moreover, the agaricomycetes *Coprinopsis cinerea* and *Schizophyllum commune* are model organism that widely used for studying sexual development in mushrooms (Specht, 1995; Inada et al., 2001). Thus, research on mushroom biology and biotechnology in agaricomycetes may contribute to solve food, health, and environmental problems.

In *Agaricomycetes*, the gilled mushrooms in the genus *Pleurotus* are composed of more than 200 species (Corrêa et al., 2016). Among them, *P. ostreatus* has developed economical prominent in the aspect of important cultivated mushrooms (Gregori et al., 2007; Corrêa et al., 2016). In 2020, the market value of United States mushroom crop including *P. ostreatus* was 1.15 billion USD (NASS-USDA, 2020). Furthermore, this species is a white-rot fungus that widely used for genetic, transcriptomics and proteomics studies of lignin biodegradation (Salame et al., 2012; Nakazawa et al., 2017) because various molecular genetic tools and relatively well-annotated genome databases are available (Honda et al., 2000; Nakazawa et al., 2016b; Lee et al., 2020). Thus, this fungus is worth studying because of their benefits and market values.

Classical breeding in mushrooms has been performed for improving the cultivated strains with better properties for example high yield and good quality of crops. In mushrooms, different patterns of mating system (bipolar and tetrapolar) are presented depend on species. Most cultivated mushrooms for example *P. ostreatus*, *S. commune*, and *L. edodes* have a tetrapolar mating system, which is controlled under unlinked *A* and *B* mating-type loci (Raudaskoski and Kothe, 2010; Kües et al., 2011). Some edible mushrooms such as *Pholiota nameko* (nameko) and *Auricularia auricula-judae* (black wood ear) have a bipolar mating system, which is controlled by only one locus (Kües et al., 2011). However, breeding of mushrooms is difficult to manipulate due to the complicated mating types, and the limited knowledge on mushroom breeding when compared to plant or animal breeding (Chakravarty, 2011; Sonnenberg et al., 2008). Furthermore, identifying hybrid strain for example in *A. bisporus* using the typical breeding is laborious and take a long time (Chakravarty, 2011). Therefore, improvement of methodologies for straightforward and efficient breeding are necessary.

To find a solution for the problems related with classical breeding, molecular breeding including molecular marker-assisted selection and genomic selection, has been applied. Furthermore, a gene targeting technique using homologous recombination was used to modify an endogenous gene. However, homologous recombination rarely occurs in some agaricomycetes due to high activity of non-homologous end joining (NHEJ), which controls under the Ku70/Ku80 heterodimer (Nakazawa et al. 2011b; Salame et al. 2012). In wild type strain of *P. ostreatus*, a versatile-peroxidase-encoding gene (*mnp4*, parenthesis *vp1*) disruptant strain was obtained with very low efficiency (2.3%). After that an efficient gene disrupting system was established in *P. ostreatus* Δ*ku80,* which is a NHEJ-deficient strain named 20b (Salame et al. 2012). Therefore, mushroom strains with desired phenotype(s) could be more efficiently generated using gene targeting. However, introduction of a foreign DNA or a transgene into mushrooms using a gene targeting technique can be defined as a genetically modified (GM) mushrooms. Many consumers worry about biomedical risks and environmental side effects of the GM organisms. In USA, many GM crops were approved by the Food and Drug Administration (FDA) such as tomato with delayed ripening, and cotton and soybeans with resistant to herbicides. Moreover, a great number of GM varieties including carrots, eggplants, potatoes, and strawberries are available in the market also (Zhang et al. 2016). In EU, 19 out of 27 countries fully or partially banned GMO. For example, the Netherlands, Austria, and Italy have selected a total ban, while Romania and England are open to GMOs (European commission, 2021). Therefore, to generate mushroom strains that

can be more widely accepted in many countries, new tools for the molecular breeding of non-GM mushrooms are required.

The clustered regularly interspaced short palindromic repeat (CRISPR) is a family of DNA sequences identified in around 90% and 50% of archaea and bacteria genomes, respectively (Hille et al., 2018). Cas9 (CRISPR-associated protein 9) is an endonuclease that uses a 20-bp of single-guided RNA (sgRNA) as a guide to recognize and cleave a specific site on double-stranded DNA (Ran et al., 2013). CRISPR/Cas9 system was first discovered as an adaptive immune system against viruses in prokaryotes (Ishino et al., 1987; Jinek et al., 2012). Nowadays, this technique can be used for genome engineering to efficiently and specifically modify targeted gene on the chromosome. In agaricomycetes, CRISPR/Cas9 has been applied for an efficient gene targeting approach in *C. cinerea* and *G. lucidum*; however, CRISPR/Cas9 plasmid was integrated into the host chromosomes (Qin et al. 2017; Sugano et al. 2017; Wang et al. 2020). An alternative way to perform CRISPR/Cas9 genome editing is to use a pre-assembled Cas9 ribonucleoprotein (Cas9 RNP) complex and *in vitro* transcribed sgRNA. The main advantage of this Cas9 RNP is the low off-target effects, and plasmid will not be incorporated into the host genome (Kim et al., 2014; Ramakrishna et al., 2014). Thus, this approach may be helpful for molecular breeding because it can be applied for gene targeting without introduction of non-endogenous DNA sequence.

The aim of this thesis is to establish CRISPR/Cas9-assisted genome editing in *P. ostreatus*. Using plasmid-based CRISPR/Cas9, the author demonstrated an efficient gene mutation and replacement using NHEJ and HDR, respectively. The effects of *pcc1* and *clp1*, which involved in sexual development in *P. ostreatus*, were also clarified with this technique. Furthermore, combination of Cas9 RNP with split-marker approach to overcome disadvantages of NHEJ-deficiency strain were developed. This is the first work on genome editing using CRISPR/Cas9 in an edible mushroom *P. ostreatus*, which is a first step for molecular breeding of non-GM edible mushrooms.

In the chapter 1, the author attempted efficient gene mutagenesis using plasmid-based CRISPR/Cas9 in *P. ostreatus*. Plasmids harboring expression cassettes of Cas9 and different single guide RNAs targeting *fcy1* or *pyrG* were individually transferred into fungal protoplasts of the PC9 strain, which generated some strains exhibiting resistance to 5-fluorocytosine and 5-fluoroorotic acid, respectively. Genomic PCR followed by sequencing revealed small insertions/deletions or insertion of a fragment derived from a plasmid at the target site in some of the drug-resistant strains. Furthermore, a mutation in *fcy1* via homology-directed repair using this CRISPR/Cas9 system was also efficiently introduced, which could be applied not

only for precise gene disruption, but also for insertions leading to heterologous gene expression in this fungus. This chapter demonstrates an efficient CRISPR/Cas9-assisted genome editing in *P. ostreatus*.

In the chapter 2, phenotypic analyses of *P. ostreatus pcc1* and *clp1* (*Popcc1* or *Poclp1*) mutants generated using CRISPR/Cas9 were performed. Plasmids with Cas9 expression cassette and different single guide RNAs targeting *Popcc1* or *Poclp1* were individually introduced into a monokaryotic *P. ostreatus* strain PC9 to obtain the disruptants. Unlike in *C. cinerea*, the pseudoclamp cell was not observed in monokaryotic *Popcc1* mutants, but it was observed after crossing two compatible strains with *Popcc1* mutations. In *Poclp1* mutants, dikaryosis was impaired as clamp cells were not observed after crossing, suggesting that *Po*Clp1 may be essential for clamp cell formation, like in *C. cinerea*. This chapter provides experimental results with respect to conserved and diverse mechanisms underlying sexual development in *Agaricomycetes* (at least between *C. cinerea* and *P. ostreatus*).

In the chapter 3, the author performed gene mutagenesis using Cas9 RNP in *P. ostreatus.* The pre-assembled Cas9/sgRNA targeting *pyrG* was introduced into protoplasts of a wild-type monokaryotic *P. ostreatus* strain PC9, which resulted in a generation of strains exhibiting resistance to 5-FOA. Small insertions/deletions at the target site were identified using genomic PCR followed by sequencing. The results showed Cas9 RNP-assisted gene mutagenesis can be applied for the molecular breeding in *P. ostreatus* and, possibly, in other edible mushroom strains. Furthermore, gene disruption via split-marker recombination using the Cas9 RNP system was also successfully demonstrated in the wild-type *P. ostreatus*. This chapter provides the methods that could overcome the disadvantages of NHEJ-deficiency in conventional studies with gene targeting, and also difficulty in gene targeting in various non-model agaricomycetes. Moreover, it showed that the RNP-dependent CRISPR/Cas9 would contribute to the molecular breeding of non-GM cultivated strains in the future.

Chapter 1

Development of genome editing in *Pleurotus ostreatus* using CRISPR/Cas9

INTRODUCTION

One-fifth of the discovered fungal species are grouped in the *Agaricomycetes* clade from *Basidiomycota* (Kirk et al., 2008). Some of these fungi form relatively large multicellular structures such as fruiting bodies or mushrooms for their sexual reproduction, which in some cases have been utilized as human foods for many years. Examples of famous edible mushrooms in the *Agaricomycetes* are *Pleurotus ostreatus* (oyster mushroom), *Agaricus bisporus* (champignon), and *Boletus edulis* (porcini). Furthermore, in terms of medicinal properties, *A. bisporus* has been suggested to cure microbial diseases and cancer (Bhushan and Kulshreshtha, 2018). An aqueous extract of *Lentinula edodes* (shiitake) could inhibit human breast cancer and stimulate the immune system (Israilides et al., 2008). Thus, fungi from the *Agaricomycetes* class are worth studying because of their many advantages to humans.

To generate highly valuable strains of cultivated mushrooms, breeding has been conducted (Sonnenberg et al., 2008; Chakravarty et al., 2011). The typical breeding of cultivated mushrooms uses strain crossing to modify the characteristics of the organisms; useful traits from a specific donor are introduced into an acceptor to yield a new hybrid strain with better properties. However, to restore the quality of a commercial strain, repeated back-crossing is required (Sonnenberg et al., 2008), which is laborious and takes a long time. Moreover, most of the cultivated mushrooms have a tetrapolar mating system ruled by two unlinked mating loci *A* and *B* (Raudaskoski and Kothe, 2010; Kües et al., 2011), which complicates crossing and breeding. Therefore, new methodologies for more efficient and simpler breeding are needed.

Molecular genetics may resolve the problems associated with classical breeding. Indeed, molecular marker-assisted selection was developed for mushroom breeding (Okuda et al., 2009; Dai et al., 2017). Furthermore, an efficient gene targeting method for gene disruption or modification using homologous recombination is available in *P. ostreatus* (Salame et al., 2012), one of the most economically important cultivated mushrooms (Gregori et al., 2007; Corrêa et al., 2016), and a white-rot fungus frequently used for molecular genetic studies on lignin degradation (Salame et al., 2013; Nakazawa et al., 2017; Yoav et al., 2018). Using this system, molecular breeding could be conducted to generate strains with desired phenotype(s) much more quickly and efficiently. Recently, the generation of sporeless strains by disrupting *mer3* or *msh4* with this system was shown (Yamasaki et al., 2021). However,

such strains are considered genetically modified organisms (GMOs) because of the introduction of non-endogenous DNA sequence(s). In many European and Asian countries, GM crops are fully or partially banned. For example, product mixtures with specific percentages of GMO crops are acceptable in some countries such as the Czech Republic and Spain (Mahaffey et al., 2016), while all the GMO crops are prohibited in Germany and France. Therefore, to generate cultivated strains that are more easily accepted by the societies, new tools for the molecular breeding of non-GM mushrooms need to be developed.

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9), which is an adaptive immune system found in archaea and bacteria (Ishino et al., 1987; Jinek et al., 2012; Song et al., 2019), has been recently utilized as a versatile gene-targeting tool. The Cas9 endonuclease is guided to a targeted chromosome site by a 20-bp single guide RNA (sgRNA), which results in the cleavage of genomic DNA at a specific site on the chromosome, followed by non-homologous end joining (NHEJ)-mediated repair. This sometimes introduces mutations at the target site due to errors in the repair process. Introducing the Cas9-sgRNA ribonucleoprotein (RNP) complex into mushroom strains would allow targeted gene mutagenesis without the introduction of non-endogenous DNA sequence for efficient molecular breeding. In 2016, it was reported that the well-known white button mushroom (*Agaricus bisporus*) with a CRISPR/Cas9 edited genome escaped GMO regulation of USDA in a news article of Nature (Waltz, 2016). Thus, this system can be used to generate new-type of non-GM mushroom strains that can be more readily accepted. In this study, the author demonstrates efficient CRISPR/Cas9-assisted gene mutagenesis in *P. ostreatus* by introducing a plasmid with Cas9 and sgRNA, which is a first step toward molecular breeding of non-GM mushrooms using CRISPR/Cas9.

MATERIALS AND METHODS

Strains, culture conditions, and genetic techniques

Pleurotus ostreatus monokaryon strain PC9 (Spanish Type Culture Collection accession number CECT20311), and 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 was used for routine cultures. The cultures were maintained at 28°C under continuous darkness, unless otherwise stated. To grow uracil and uridine auxotrophic strains, 0.18 mM uracil and 20 mM uridine were added to YMG medium (YMGUU), if necessary. Either 0.1% (w/v) 5-FC or 5-FOA was added to the medium when necessary.

The transformation of *P. ostreatus* strains using the hygromycin resistance gene (*hph*) was performed using protoplasts prepared from mycelial cells as described by Salame et al. (2012).

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

Strain	Genotype/description	Source
PC9	*A2B1*/5-FC and 5-FOA-sensitive (Nakazawa and Honda, 2015; Nakazawa et al. 2016b)	Larraya et al. (1999)
20b	*A2B1 ku80*::*Cbx*^{R}/5-FC- and 5-FOA-sensitive	Salame et al. (2012)
fc0-3	*A2B1 fcy1-1*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg1	This study
fc1-3	*A2B1 fcy1-2*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2	This study
fc1-4	*A2B1 fcy1-3*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2	This study
fc1-5	*A2B1 fcy1-4*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2	This study
fc2-1	*A2B1 fcy1-5*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2	This study
fc2-2	*A2B1 fcy1-6*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2	This study
py1-2	*A2B1* /a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
py1-4	*A2B1 pyrG-3*/a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
py1-6	*A2B1 pyrG-4*/a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
py1-9	*A2B1* /a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
py1-10	*A2B1 pyrG-5*/a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
py1-14	*A2B1 pyrG-6*/a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
py1-17	*A2B1 pyrG-7*/a 5-FOA-resistant strain obtained after introducing pCcPef3-126-*pyrG*sg1	This study
hr1	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr2	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr3	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr4	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr5	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 1 kb	This study
hr6	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 1 kb	This study
hr7	*A2B1 ku80*::*Cbx*^{R}/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.2 kb	This study
hr8	*A2B1 ku80*::*Cbx*^{R}/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr9	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr10	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr11	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 1 kb	This study
hr12	*A2B1 ku80*::*Cbx*^{R}/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.2 kb	This study
hr13	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr14	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr15	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 0.5 kb	This study
hr16	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 1 kb	This study
hr17	*A2B1 ku80*::*Cbx*^{R} *fcy1-7*/a 5-FC-resistant strain obtained after introducing pCcPef3-126-*fcy1*sg2 and donor DNA with homology arms of 1 kb	This study

Design of sgRNAs targeting *fcy1* or *pyrG*

The different sgRNA sequences used to target *fcy1* and *pyrG* (Nakazawa et al., 2016b) [Protein ID: 89004 and 83414, respectively, in the genome database of *P. ostreatus* PC9 (https://mycocosm.jgi.doe.gov/PleosPC9_1/PleosPC9_1.home.html)] were designed based on on-target (Doench et al., 2014) and off-target (Xiao et al., 2014) scores calculated by the Focas UI website (http://focas.ayanel.com). The four sgRNA sequences were: *fcy1*sg1 (*fcy1*, nucleotide positions 32–51) and *fcy1*sg2 (*fcy1*, 92–111); *pyrG*sg1 (*pyrG*, 521–540) and *pyrG*sg2 (*pyrG*, 559–578), from the start codon (Table S1).

Plasmid construction

In this study, the vector pCcPef3-126 was used for genome editing (Fig. S1) (Sugano et al., 2017; Nguyen et al., 2020). The basidiomycete codon optimized *Streptococcus pyogenes* Cas9 for with 3 × nuclear localization sequences (NLSs) and the sgRNA scaffold were driven by the *C. cinerea ef3* (an elongation factor 3) promoter and the *u6* promoter, respectively. The *hph* cassette from pPHT1 (Cumming et al., 1999) was used to confer resistance to hygromycin B on *P. ostreatus*. The double-stranded DNA, which was prepared by annealing two DNA oligonucleotides [primers CI19/CI20 (*fcy1*sg1) and CI21/CI22 (*fcy1*sg2) for *fcy1*; TB41/TB42 (*pyrG*sg1) and TB43/TB44 (*pyrG*sg2) for *pyrG* (Table S1)], containing each sgRNA sequence was inserted into the *Bsa*I site of the linearized pCcPef3-126 vector using Golden Gate assembly (Engler et al., 2008; 2009). The sgRNA insertion into plasmids was verified with Sanger sequencing. The resulting plasmids were named pCcPef3-126-*fcy1*sg1, pCcPef3-126-*fcy1*sg2, pCcPef3-126-*pyrG*sg1, and pCcPef3-126-*pyrG*sg2, respectively.

Design of the donor DNA templates for homologous recombination

For precise gene replacement using homologous recombination in the *fcy1* gene, donor DNA templates (with a 25-bp deletion of the *fcy1*sg2 sequence) with homology arms of 1 kb, 0.5 kb, or 0.2 kb, were constructed using overlap extension PCR. Approximately 1-kb genomic fragments containing 5'-upstream and 3'-downstream sequences were amplified using CI50/CI51 and CI52/CI53, respectively (Fig. 1A). These fragments were fused by CI50/CI53 to obtain the intact donor DNA, followed by amplification with TB11/TB12 and TB9/TB10 to reduce the length of homologous arms to 0.5 kb and 0.2 kb, respectively (Fig. 1B). The resulting fragments were designated as donor DNA with homology arms of 1 kb (2071 bp in total), 0.5 kb (1008 bp in total), and 0.2 kb (407 bp in total), respectively (Table S2).

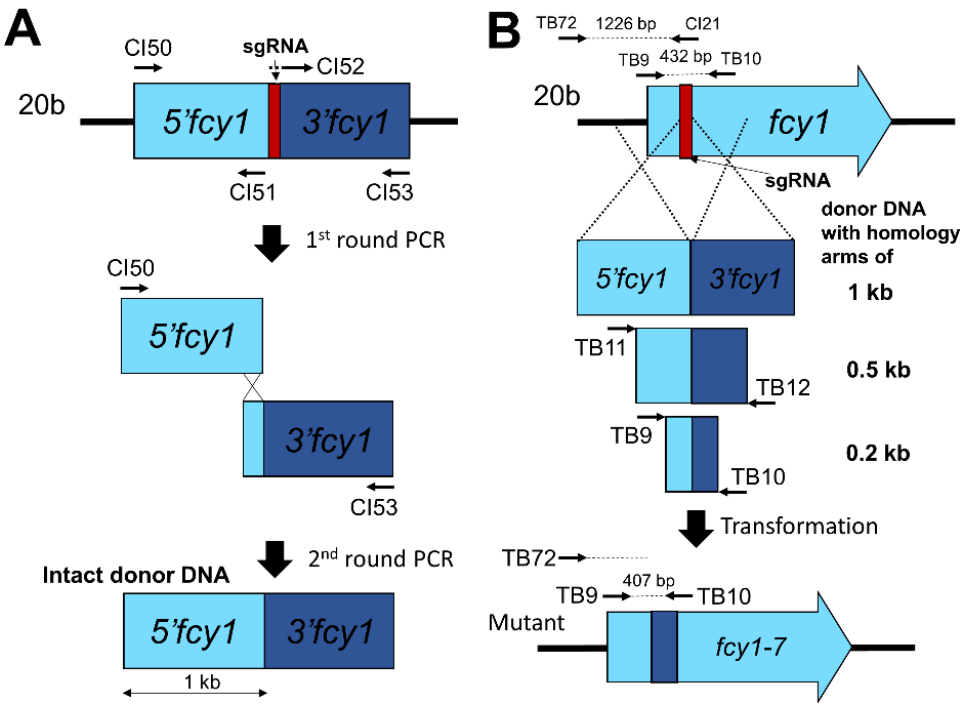

Fig. 1. Targeted gene replacement using homologous recombination along with CRISPR/Cas9. (A) A schematic diagram of the procedure used to construct the donor DNA template that lacks the sgRNA sequence for homologous recombination. (B) A schematic diagram showing the method for *fcy1* mutation using CRISPR/Cas9 and donor DNA. PCR fragments with around 1 kb, 0.5 kb, and 0.2 kb each of right and left homologous arms were used as donor DNA templates. Black arrows display the primer pairs used for the PCR experiments in Fig. S2C.

Rapid colony PCR

To verify gene mutations, rapid colony PCR was performed. Genomic DNA was extracted with the thermolysis method described by Zhang et al. (2010) with some modifications. Briefly, a small agar plug (approximately 3×3 mm) covered with mycelium was placed into 200 µl of sterilized distilled water for removal of PCR inhibitors and briefly centrifuged at 13,200 × *g* for 1 min. Then, 200 µl of DNA extraction buffer [50 mM sodium phosphate (pH 7.4), 1 mM EDTA, and 5% (v/v) glycerol] was added, the mixture was incubated at 85°C for 30 min, and then kept at −20°C until further use. In this study, the author used the EmeraldAmp MAX PCR Master Mix (Takara Bio, Shiga, Japan) and KAPA2G Robust HotStart PCR kit (Nippon Genetics, Tokyo, Japan) for the PCR experiments.

Genomic PCR to identify mutations

To examine the type of mutations, long-range PCR was performed. The high-quality fungal genomic DNA was extracted with the CTAB method (Zolan and Pukkila, 1986; Muraguchi et al., 2003). Briefly, *P. ostreatus* strains were grown on YMG agar medium for 7

days and the mycelial cells were freeze-dried overnight using a small freeze-dryer (FDS-1000, EYELA, Tokyo, Japan). The disrupted lyophilized cells were resuspended in 900 µl of CTAB buffer [1% (w/v) CTAB, 0.68 M NaCl, 50 mM Tris-HCl pH 8.0, and 10 mM EDTA] with 18 µl of 2-mercaptoethanol and incubated at 50°C for 30 min. Next, 900 µl of chloroform was added, mixed by inversion, and then centrifuged at 13,200 × *g* for 5 min. The supernatant was mixed with chloroform and phenol (2:1:1), and centrifuged. Then, 600 µl of isopropanol and 30 µl of 5 M NaCl were added to the supernatant. The mixture was centrifuged at 13,200 × *g* for 15 min, and the DNA pellet was washed once with 1 ml of cold 70% ethanol, air dried, and resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) at a final concentration of 10 µg/ml of RNase A. In this study, the author used the Proofreading polymerase KOD FX Neo (Toyobo, Osaka, Japan) with a step-down cycle, as indicated by the manufacturer for long-distance PCR amplification. Then, the PCR fragments were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), followed by Sanger sequencing.

RESULTS

Expressing Cas9 and sgRNA by plasmid introduction frequently confers resistance to 5-FC and 5-FOA on the *P. ostreatus* PC9 strain

To examine the efficiency of CRISPR/Cas9-assisted gene mutagenesis by introducing a plasmid expressing Cas9 and sgRNA, the *fcy1* and *pyrG* genes were selected as targets to be mutated because their single-gene mutations confer resistance to 5-FC and 5-FOA in *P. ostreatus*, respectively (Nakazawa et al., 2016b). This allowed us to identify the mutants easily and efficiently by examining resistance/sensitivity. The plasmids pCcPef3-126-*fcy1*sg1, pCcPef3-126-*fcy1*sg2, pCcPef3-126-*pyrG*sg1, and pCcPef3-126-*pyrG*sg2 were separately introduced into the *P. ostreatus* PC9 host strain (Table 1) to obtain hygromycin-resistant transformants. These plasmids express *fcy1*- or *pyrG*-targeting sgRNAs (*fcy1*sg1, *fcy1*sg2, *pyrG1*sg1, and *pyrG*sg2), respectively, together with Cas9 and a hygromycin phosphotransferase (Hph) that confers resistance to hygromycin B. The empty vector pCcPef3-126, which expresses an sgRNA scaffold without the targeting sequence, was also introduced as a control. A total of 14 hygromycin-resistant transformants were obtained in three independent experiments by introducing the empty vector, all of which did not grow on YMG with 0.1% (w/v) of 5-FC or 5-FOA (Table 2). This result showed that introducing the empty vector pCcPef3-126 rarely confers resistance to 5-FC and 5-FOA on *P. ostreatus* PC9. In contrast, one out of five (20%) and 22 out of 28 (78.6%) hygromycin-resistant transformants,

obtained by introducing pCcPef3-126-*fcy1*sg1 and pCcPef3-126-*fcy1*sg2, respectively, exhibited resistance to 5-FC. Eight out of 17 (47.1%) and two out of seven (28.6%) hygromycin-resistant transformants, obtained by introducing pCcPef3-126-*pyrG*sg1 and pCcPef3-126-*pyrG*sg2, respectively, exhibited resistance to 5-FOA. Similar results were obtained in the replicate experiments (Table 2). These results suggested that 5-FC- or 5-FOA-resistant *P. ostreatus* strains can be frequently generated by introducing plasmids using the CRISPR/Cas9 system; the frequency/efficiency seemed to depend on the target sequence in sgRNA.

Table 2. Comparison of transformants and mutation frequency obtained using different sgRNAs targeting *fcy1* or *pyrG*.

Plasmid with sgRNA	Hygromycin-resistant strains			5-FC- or 5-FOA-resistant strains			*fcy1* or *pyrG* mutants by PCR[1]		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
Empty vector	2	4	8	0[2]	0[2]	0[2]	-	-	-
***fcy1* sgRNA No. 1**	5	11	18	1(20%)[3]	0	0	1/1	-	-
***fcy1* sgRNA No. 2**	28	23	34	22(78.6%)[3]	15(65.2%)[3]	30(88.2%)[3]	5/5	4/5	4/5
***pyrG* sgRNA No. 1**	17	19	-	8(47.1%)[3]	18(94.7%)[3]	-	7/8	10/10	-
***pyrG* sgRNA No. 2**	7	7	-	2(28.6%)[3]	2(28.6%)[3]	-	2/2	2/2	-

[1]The number of mutants which can be identified by difference size, or no amplified, bands compared to wild-type control were observed in the genomic PCR experiment (Fig. S2A and B); [2]The number of 5-FC- or 5-FOA-resistant strains; [3]Percentage of 5-FC- or 5-FOA-resistant strains from the total number of hygromycin-resistant strains.

Identification of small deletion mutations in *fcy1* and *pyrG*

Next, the author verified that the obtained 5-FC- and 5-FOA-resistant strains were *fcy1* and *pyrG* mutants, respectively. An NHEJ-mediated gene mutation using the CRISPR/Cas9 system typically produces small insertions or deletions (indels), such as the insertion of several nucleotides and deletion from a single nucleotide to several hundreds of nucleotides at/around a target site. Therefore, the author first attempted to PCR-amplify genomic fragments of the partial open reading frames (ORFs) of *fcy1* (432 bp) and *pyrG* (356 bp) containing the target sequences of sgRNA using primer pairs TB9/TB10 and TB53/TB54, respectively. The primer pair FY15/FY16, which amplifies a 430-bp genomic fragment from the ORF of *mer3* encoding an ATP-dependent DNA helicase (Protein ID 82484 in the JGI genome database) was also used as a positive control.

Some 5-FC- or 5-FOA-resistant strains were randomly selected for examination of gene mutation using genomic PCR (Table 2), and the result of agarose gel electrophoresis in ten strains each of 5-FC and 5-FOA resistance was shown in the supplementary figure (Fig. S2A

and B, respectively). The PCR product was amplified from the ten 5-FC-resistant strains and the ten 5-FOA-resistant strains used (lanes 1–10 in Fig. S2A and B) when the primer pair FY15/FY16 was used. As shown in Fig. S2A and B, similar to the parental control strain PC9, the fragment was amplified from two out of ten 5-FC-resistant strains (lanes 4 and 6 in Fig. S2A; strains fc1-4 and fc2-1, respectively) and one out of ten 5-FOA-resistant strains (lane 8 in Fig. S2B; strain py1-17) when the primer pairs TB9/TB10 and TB53/TB54 were used, respectively. The sizes of the fragments amplified from fc1-4 and fc2-1 seemed to be different from that amplified from PC9 (Fig. S2A), suggesting that small indels may have been introduced at the target sites of *fcy1*/*pyrG*, at least in fc1-4 and fc2-1.

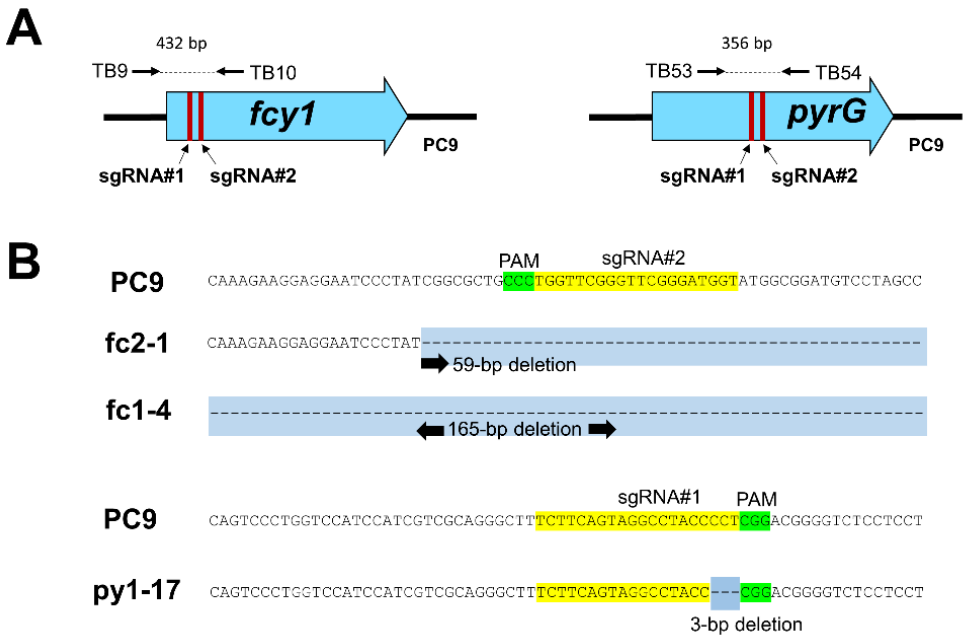

Fig. 2. Identification of *fcy1* and *pyrG* mutations in some strains. (A) A schematic diagram of the *fcy1* and *pyrG* loci in the PC9 host strain with sgRNA recognition sites. The dash lines indicate the region amplified by genomic PCR. Black arrows display the primer pairs used for the PCR experiments in Fig. S2A and B. (B) DNA sequencing to identify small indel mutations in *fcy1* or *pyrG* mutants. For highlights in the nucleotide sequence: yellow shades indicate sgRNA, green shades indicate a protospacer adjacent motif (PAM) sequence, and dash lines indicate deletion.

To confirm if small indels were introduced at the target sites in the three resistant strains from which the genomic fragment was amplified (fc1-4, fc2-1, and pyl-17), the nucleotide sequences of the PCR-amplified fragments were analyzed using TB9 or TB10 for *fcy1*, and TB53 or TB54 for *pyrG*. The results revealed 165-bp and 59-bp deletions around the target site of *fcy1*sg2 in fc1-4 and fc2-1, respectively. A 3-bp deletion mutation at the target site of *pyrG*sg1 in the 5-FOA resistant strain, py1-17 (lane 8), was also revealed (Fig. 2B; Table 1). The mutations newly identified in fc1-4, fc2-1, and py1-17 were designated as *fcy1-3*,

fcy1-5, and *pyrG-7*, respectively (Table 1). These results indicated that they are *fcy1* or *pyrG* mutants harboring small indel mutations; however, the mutations were not identified in many other strains from which the genomic fragment was not amplified.

Identification of the CRISPR/Cas9 plasmid insertion at the target sites

Considering the fact that the PCR fragment was not amplified from many 5-FC- or 5-FOA-resistant strains (lanes 1–3, 5, 7–10 in Fig. S2A; lanes 1–7, 9–10 in Fig. S2B), the mutations could have been introduced into the chromosomes of these strains in different manners. The author hypothesized that the introduced plasmids had been inserted at the target site of each sgRNA. Based on this assumption, eight pairs of primers were designed to hybridize to the genomic region, which is located approximately 200-bp apart from the sgRNA target site in the first primer (primer TB9 or TB10 for *fcy1*; TB53 or TB54 for *pyrG*), and the *C. cinerea β1-tubulin* promoter or terminator in the plasmids in the other primer (primer TN40 or TN46). The set of primers TB9/TN40, TB9/TN46, TB10/TN40, and TB10/TN46 for *fcy1,* and TB53/TN40, TB53/TN46, TB54/TN40, and TB54/TN46 for *pyrG*, were used for genomic PCR on six 5-FC- and six 5-FOA-resistant strains, respectively (Table S2). In the case of *fcy1*, around 6 kb, 1.5 kb, 7 kb, and 10 kb fragments were amplified from four 5-FC-resistant strains (namely fc0-3, fc1-3, fc2-2, and fc1-5, respectively) when the primer pairs TB10/TN40, TB10/TN46, TB9/TN40, and TB9/TN46 were used, respectively. In the case of *pyrG*, around 1.7 kb, 2.5 kb, 2.5 kb, 5 kb, 8 kb, and 6 kb fragments were amplified from six 5-FOA-resistant strains (namely py1-2, py1-4, py1-6, py1-9, py1-10, and py1-14, respectively) when the primer pairs TB53/TN46, TB53/TN46, TB53/TN46, TB53/TN46, TB54/TN46, and TB53/TN46, were used, respectively. These PCR fragments were not amplified from their parental control strain, PC9, when these sets of primers were used (data not shown). These results suggested that the introduced plasmids had been inserted at the target sites of *fcy1*/*pyrG,* at least in the ten strains from which the fragment was amplified.

To confirm the possibility of the insertional mutation, the PCR-amplified fragments were subjected to DNA sequencing using primers TB9 or TB10 for *fcy1*, and TB53 or TB54 for *pyrG*. The results demonstrated the insertion of the introduced plasmids at the target sites of the three 5-FC-resistant strains (strain fc0-3 generated by introducing the pCcPef3-126-*fcy1*sg1, and strains fc1-3 and fc2-2 by pCcPef3-126-*fcy1*sg2), and the plasmid insertion altogether with a 1 bp insertion at the target site of *fcy1*sg2 in one 5-FC-resistant strain, fc1-5 (Fig. 3A). As for *pyrG* mutations in the 5-FOA-resistant strains, the mutations were successfully identified in the four mutants. The insertion of the introduced plasmid at the

target site of *pyrG*sg1 in the three 5-FOA-resistant strains, namely py1-4, py1-6, and py1-14, and the plasmid insertion altogether with a 2 bp insertion at the target site of *pyrG*sg1 in the one 5-FOA-resistant strain, py1-10, are shown in Fig. 3B. The author also attempted to identify the insertional mutations of the two 5-FOA-resistant strains, py1-2 and py1-9; however, the PCR-amplified fragments could not be sequenced using the TB53 primer. The mutations in these strains, namely fc0-3, fc1-3, fc1-5, fc2-2, py1-4, py1-6, py1-10, and py1-14, were designated as *fcy1-1*, *fcy1-2*, *fcy1-4, fcy1-6,* and *pyrG-3–6,* respectively (Table 1). These results suggested that the insertional mutation was introduced into *fcy1* and *pyrG* in some of the 5-FC- and 5-FOA-resistant strains, respectively.

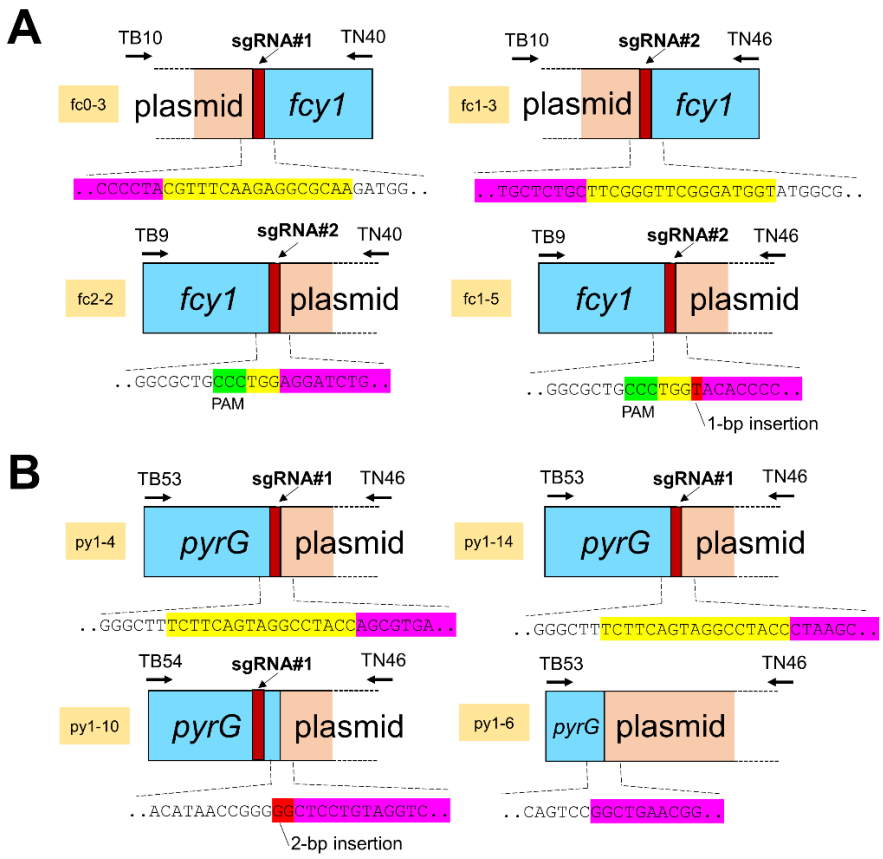

Fig. 3. Identification of insertional mutations in some *fcy1* or *pyrG* mutants. Schematic diagrams showing how the introduced plasmids had been inserted at the target sites of *fcy1* (A) or *pyrG* (B). For highlights in the nucleotide sequence: yellow shades indicate sgRNA, green shades indicate PAM sequence, pink shades indicate plasmid sequence, and red shades indicate insertion.

Targeted gene replacement using homologous recombination with a donor DNA template

The gene mutations identified in the above experiments were most likely introduced by NHEJ after the double-strand DNA break (DSB), which was caused by the expressed Cas9-sgRNA complexes. However, the NHEJ-mediated mutations are generally unpredictable,

which precludes us from generating strains with the desired mutations using CRISPR/Cas9. For precise gene replacement and introduction of the targeted mutation, CRISPR/Cas9 with a donor DNA template may be used to induce homology-directed repair (HDR)-mediated mutations in *P. ostreatus*. First, to examine whether the ORF of *fcy1* can be frequently replaced with that of donor DNA (with the 25-bp deletion in the ORF containing the *fcy1*sg2 sequence; Fig. 1A) using CRISPR/Cas9-assisted homologous recombination, the plasmid pCcPef3-126-*fcy1*sg2 was introduced with or without a donor DNA template with homology arms of 1 kb (Fig. 1B). In this experiment, strain 20b (a *ku80* disruptant from PC9), but not PC9, was used because the *ku80* deficiency impairs the NHEJ pathway and decreases the frequency of NHEJ-mediated ectopic integration (Salame et al., 2012). Four hygromycin-resistant transformants were obtained from three independent experiments by introducing only the plasmid pCcPef3-126-*fcy1*sg2, all of which did not grow on YMG with 0.1% (w/v) 5-FC (Table 3). This result indicated that introducing the plasmid pCcPef3-126-*fcy1*sg2 alone rarely confers resistance to 5-FC on the *P. ostreatus* 20b strain, a result inconsistent with that of the wild-type strain, as introducing pCcPef3-126-*fcy1*sg2 onto PC9 frequently conferred resistance to 5-FC (Table 2). This may be due to our hypothesis that the introduced plasmid was frequently inserted at the target site by NHEJ in PC9, but not in 20b due to its reduced NHEJ activity/pathway. Five out of six (83.3%) hygromycin-resistant transformants, obtained by introducing pCcPef3-126-*fcy1*sg2 along with the donor DNA template, exhibited resistance to 5-FC (Table 3). This result suggested that targeted gene replacement may occur frequently when the CRISPR/Cas9 plasmid is introduced into *P. ostreatus* 20b strains along with a DNA repair template.

Second, to examine the effect of homology arm length on frequency/efficiency, the plasmid pCcPef3-126-*fcy1*sg2 with reduced size of DNA repair templates (homology arms of 0.5 kb and 0.2 kb) was introduced into the 20b strain. As shown in Table 3, ten out of 13 (76.9%) and two out of four (50.0%) hygromycin–resistant strains, obtained in three independent experiments by introducing pCcPef3-126-*fcy1*sg2 with homology arms of 0.5 kb and 0.2 kb, respectively, exhibited resistance to 5-FC. These results suggested that the frequency of 5-FC resistance may be higher when using a longer DNA repair template with the CRISPR/Cas9 system.

The author then verified that the *fcy1* sequence was replaced with the introduced donor DNA, as expected. The 17 5-FC-resistant strains obtained by introducing the pCcPef3-126-*fcy1*sg2 plasmid along with the donor DNA template with 1-kb homology arms (five strains, hr5, hr6, hr11, hr16, hr17), 0.5-kb one (ten strains, hr1–4, hr8, hr9, hr10, hr13–15), or 0.2-kb

one (two strains, hr7 and hr12), were used for this experiment (Table 1). First, the author examined whether a genomic fragment (1226 bp) was amplified from strains hr1–17 when the primer pair TB72/CI21 was used (Fig. 1B). This fragment was anticipated to be amplified from 20b, but not from strains into which the HDR-mediated *fcy1* mutation had been introduced, due to loss of the hybridization sequence from primer CI21 after the HDR-mediated mutation. As shown in Fig. S2C, the expected fragment was amplified from the 20b strain (lane WT), but not from the 5-FC-resistant strains (lanes 1–17). This result suggested that the HDR-mediated mutation could be introduced into the genomes of all 5-FC-resistant strains.

Furthermore, the other primer pair TB9/TB10 was used to confirm whether a 432-bp PCR product was amplified from the wild type strain, while a shorter size of genomic fragment (407 bp) was amplified from the prospect mutant strains due to loss of a region containing the sgRNA recognition site on the DNA repair template (Fig. 1B). As shown in Fig. S2C, the expected fragment was amplified from the 20b strain (lane WT), whereas the shorter fragment was amplified from 14 out of 17 5-FC-resistant strains (lanes 1–6, 9–11, 13–17; strains hr1–6, hr9–11, hr13–17, respectively). The mutations in these 14 strains were designated as *fcy1-7* (Table 1). This result indicated that all five, and nine out of ten 5-FC-resistant strains were *fcy1* disrupted when using the repair templates with homology arms of 1 kb and 0.5 kb, respectively (Table 3). Moreover, these results suggested that precise/targeted gene replacement can be performed using CRISPR/Cas9 along with a donor DNA template with at least 0.5-kb homology arms in *P. ostreatus*.

Table 3. Comparison of transformants and mutation frequency obtained using CRISPR/Cas9 and donor DNA templates.

Plasmid with sgRNA	**Donor DNA template**	**Hygromycin-resistant strains**				**5-FC-resistant strains**				***fcy1* mutant by PCR[1]**			
		Rep 1	**Rep 2**	**Rep 3**	**Total**	**Rep 1**	**Rep 2**	**Rep 3**	**Total**	**Rep 1**	**Rep 2**	**Rep 3**	**Total**
***fcy1* sgRNA No. 2**	**-**	0	2	2	4	0	0	0	0	0	0	0	0
	0.2 kb	0	2	2	4	0	1	1	2(50%)[2]	0	0	0	0
	0.5 kb	4	5	4	13	4	3	3	10(76.9%)[2]	4	2	3	9(69.2%)[3]
	1 kb	2	2	2	6	2	1	2	5(83.3%)[2]	2	1	2	5(83.3%)[3]

[1]The number of mutants which can be identified by difference size, or no amplified, bands compared to wild-type control were observed in the genomic PCR experiment (Fig. S2C);
[2]Percentage of 5-FC-resistant strains from the total number of hygromycin-resistant strains;
[3]Percentage of *fcy1* mutants from the total number of hygromycin-resistant strains.

DISCUSSION

Here, the author demonstrates efficient CRISPR/Cas9-assisted gene mutagenesis by plasmid introduction in *P. ostreatus*. In the *Agaricomycetes* class, CRISPR/Cas9-assisted genome editing was previously reported in *C. cinerea* and *Ganoderma lucidum* by plasmid introduction; however, the efficiencies/frequencies were 10.5–32.0% (Sugano et al., 2017; Wang et al., 2020), which were much lower than those in this study (20–94.9%). Therefore, the CRISPR/Cas9 system in *P. ostreatus* used in this study may currently be the most efficient among agaricomycetes. This new, efficient tool developed in *P. ostreatus* could be applied to molecular breeding as well as to studies on fruiting development and lignin degradation.

In this study, the plasmid pCcPef3-126 designed for CRISPR/Cas9 in *C. cinerea* (Sugano et al., 2017) was also available in *P. ostreatus*. Considering that the introduction of this plasmid into *P. ostreatus* PC9 resulted in the efficient introduction of gene mutations, all the expression cassettes for Cas9, Hph, and sgRNA work well not only in *C. cinerea* but also in *P. ostreatus* (both in the order *Agaricales*). Furthermore, the *hph* cassette in this plasmid, which is derived from the pPHT1 designed for hygromycin resistance transformation in *C. cinerea* (Cummings et al., 1999), was also shown to be available for that in *Ceriporiopsis subvermispora*, which belongs to the order *Polyporales* (unpublished). This suggests that CRISPR/Cas9-assisted genome editing using the pCcPef3-126 plasmid can be applied in many other agaricomycetes with high economical and medicinal value, as long as hygromycin resistance transformation is available/developed.

The author successfully identified *fcy1* or *pyrG* mutations in some of the genome-edited strains; small indels in three mutants, and insertion of the introduced plasmid in eight mutants. Small indels have been identified in genome-edited mutants of various fungi, including *C. cinerea* and *G. lucidum* (Sugano et al., 2017; Qin et al., 2017; Wang et al., 2020). However, to the best of our knowledge, the insertion of a plasmid sequence at the target site of sgRNA, which may be mediated by the NHEJ pathway, has not been previously reported, except for the intended plasmid insertion system in *Fusarium oxysporum* (Wang and Coleman, 2019). Generally, the frequency of homologous recombination in agaricomycetes is much lower than that in ascomycetes (Nakazawa et al., 2011b; Salame et al., 2012), which may be due to higher NHEJ activity in agaricomycetes. This could be the reason behind the insertional mutation in *P. ostreatus*. If this hypothesis is correct, genome-edited strains with insertional mutations may be obtained when pCcPef3-126-based CRISPR/Cas9 is performed in other agaricomycetes.

However, not all the mutations in the mutants examined in this study could be identified in the genomic PCR experiments. Therefore, the mutations might also be introduced differently. For example, the genomic sequence around the target site could be largely deleted, which would also cause the loss of the chromosomal region where the primers anneal, precluding the PCR-amplification of the fragment. Moreover, translocation is also likely to occur. These possibilities should be examined in future studies using Southern blot analyses and long-read whole-genome resequencing.

Furthermore, the author demonstrates CRISPR/Cas9-assisted gene replacement via HDR in *P. ostreatus*. Conventional gene targeting experiments using homologous recombination have been performed with *P. ostreatus* 20b strain and 1.5–2 kb homology arms (Salame et al., 2012; Nakazawa et al., 2016b), while *fcy1* mutants were successfully obtained when a shorter donor DNA template with homology arms of 1 kb and 0.5 kb was used in this study. Thus, CRISPR/Cas9-assisted replacement via HDR enables targeted gene replacement using a shorter homology arm, which may be more useful than the conventional method. Although the possibility of ectopic integration of the donor DNA as well as the introduced plasmid with the expression cassettes for sgRNA and Cas9 into the host chromosome cannot be excluded, this method could be more useful for expression cassette(s) insertion and gene knock-in/knock-out in molecular genetics studies of *P. ostreatus*.

SUMMARY

In conclusion, this is the first report demonstrating genome editing using the CRISPR/Cas9 system in an edible mushroom. Future studies will focus on developing a marker-free CRISPR system for molecular breeding of non-GM mushrooms. However, the efficiency/frequency of gene mutagenesis with Cas9/sgRNA ribonucleoprotein (RNP) complex seems to be very low in the agaricomycete *S. commune* (Jan Vonk et al., 2019), suggesting that some difficulties may have to be overcomed to establish marker-free CRISPR/Cas9 in agaricomycetes.

SUPPLEMENTARY DATA

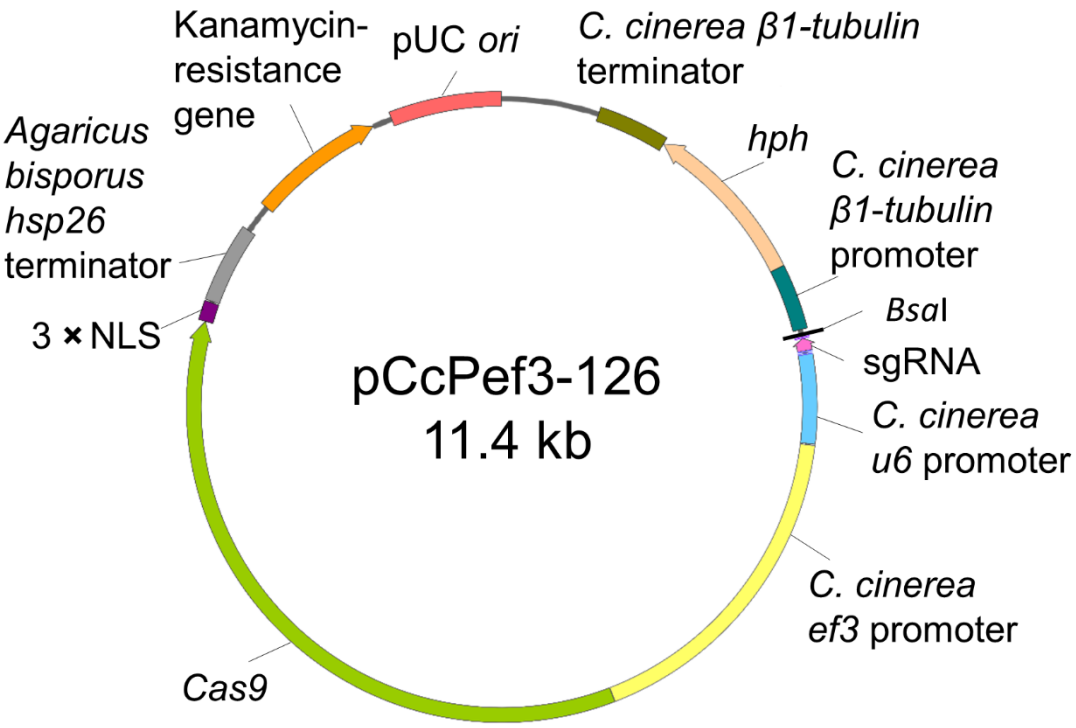

Fig. S1. A plasmid map of the pCcPef3-126 plasmid shows the components of the construct.

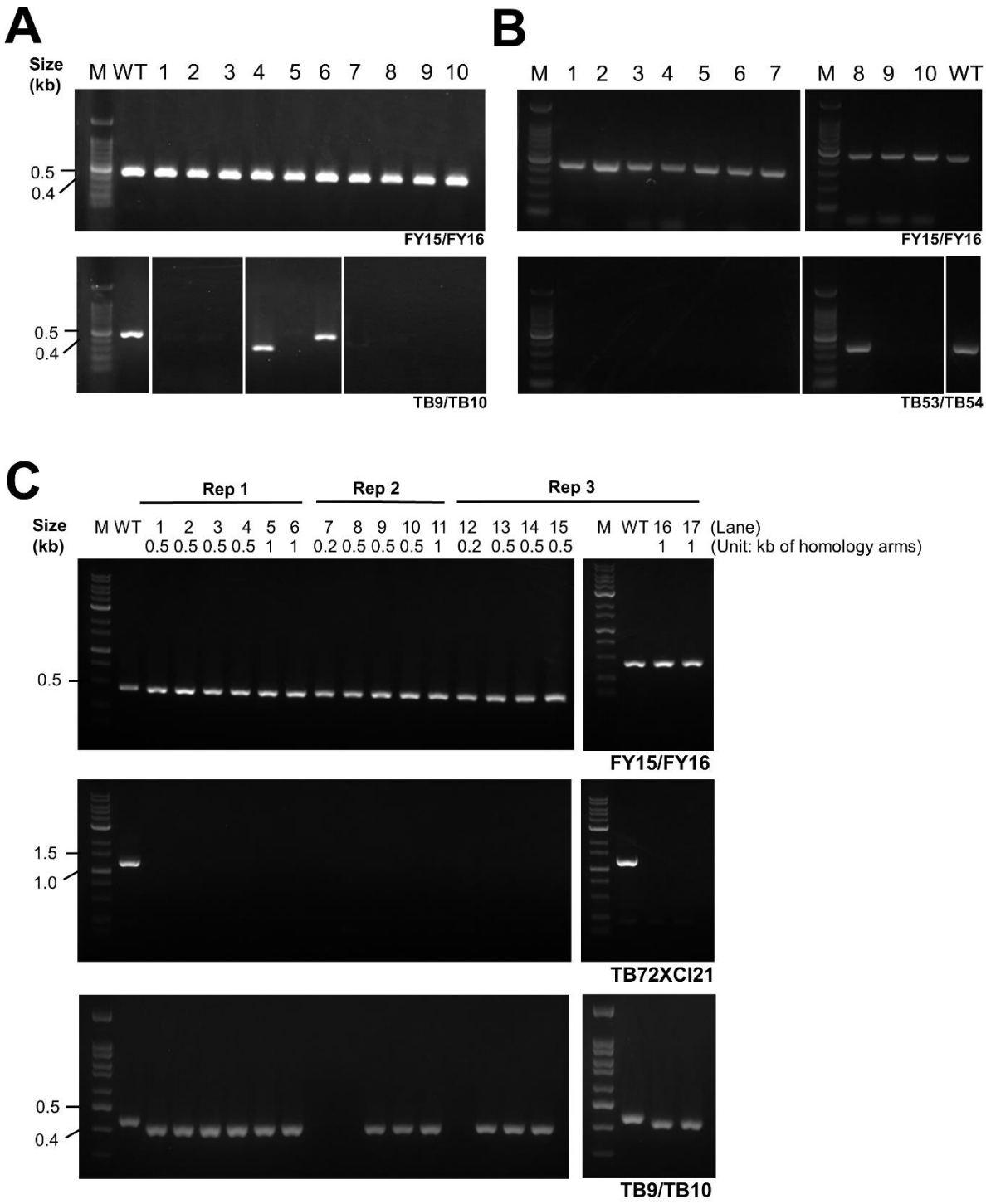

Fig. S2. Agarose gel electrophoresis. Genomic PCR experiments examining/verifying *fcy1* (A) or *pyrG* (B) mutation. (C) Genomic PCR experiments confirming gene replacement in the 5-FC-resistant strains obtained after introducing the pCcPef3-126-*fcy1*sg2 plasmid with the donor DNA templates. Lane WT, the parental strain PC9 (A and B) and 20b (C) as a positive control; Lanes 1–10 (A and B), 5-FC- and 5-FOA- resistant strains, respectively; Lanes 1–17 (C), 5-FC-resistant strains; Lane M, a 1 kb DNA ladder plus (0.1– 10.0 kb), or a 100-bp molecular weight marker (0.1–1.5 kb). For more details regarding the estimated lengths of the PCR products amplified from the genome, please see Table S2.

Table S1. Primer pairs used in this study.

Primer	**Sequence (5' to 3')**
CI19	GATTGTTGCGCCTCTTGAAACGCGA
CI20	AAACTCGCGTTTCAAGAGGCGCAAC
CI21	GATTGACCATCCCGAACCCGAACCA
CI22	AAACTGGTTCGGGTTCGGGATGGTC
CI50	TGGCGACCTGATGGTACGATG
CI51	AGCGCCGATAGGGATTCCTC
CI52	ATCCCTATCGGCGCTTGGCGGATGTCCTAGCCTCG
CI53	TGTTCGTGACGACCTCGAGG
FY15	AACCCCAAGTTCTATGCGTTG
FY16	ACTTACTCTTCTGCAGCCGAC
TB9	CATTGCTTTGGCGGTTCAAACG
TB10	GCGTACCTCAGCGTCGTGTAC
TB11	GCAGCCCTTTTCTTTCTCTTCCAGATC
TB12	CGATGTCTTCATACCATTCCTACACCG
TB41	GATTGTCTTCAGTAGGCCTACCCCT
TB42	AAACAGGGGTAGGCCTACTGAAGAC
TB43	GATTGCTTGGCAGAAATGAGCACCG
TB44	AAACCGGTGCTCATTTCTGCCAAGC
TB53	CGCCGCTTGTAGGAAACACAG
TB54	CTGCTGCCCCATACCATCTCC
TB72	CGTCACCAGTCATCGTGACC
TN40	ACCCTTTCCCCCAAAATTTGGAAGC
TN46	AAACGGCTTCACGGGCAGCC

Table S2. Estimated lengths of the PCR fragments that were amplified from each strain.

Primer set	Strain/ Function	Estimated length (bp)
CI19/CI20	*fcy1*sg1	-
CI21/CI22	*fcy1*sg2	-
TB41/TB42	*pyrG*sg1	-
TB43/TB44	*pyrG*sg2	-
FY15/FY16	Wild-type[1] / positive control	430
	Mutant / positive control	430
TB9/TB10	Wild-type[1]	432
	fcy1 mutant via NHEJ	Small indels of nucleotide, or not amplified
	fcy1 mutant via HDR	407
TB53/TB54	Wild-type[1]	356
	pyrG mutant	Small indels of nucleotide, or not amplified
TB9/TN40	Wild-type[1]	Not amplified
	fcy1 mutant	unpredictable, or not amplified
TB9/TN46	Wild-type[1]	Not amplified
	fcy1 mutant	unpredictable, or not amplified
TB10/TN40	Wild-type[1]	Not amplified
	fcy1 mutant	unpredictable, or not amplified
TB10/TN46	Wild-type[1]	Not amplified
	fcy1 mutant	unpredictable, or not amplified
TB53/TN40	Wild-type[1]	Not amplified
	pyrG mutant	unpredictable, or not amplified
TB53/TN46	Wild-type[1]	Not amplified
	pyrG mutant	unpredictable, or not amplified
TB54/TN40	Wild-type[1]	Not amplified
	pyrG mutant	unpredictable, or not amplified
TB54/TN46	Wild-type[1]	Not amplified
	pyrG mutant	unpredictable, or not amplified
CI50/CI51	5'-upstream of *fcy1*	1038
CI52/CI53	3'-downstream of *fcy1*	1048
CI50/CI53	donor DNA with homology arms of 1 kb	2071
TB11/TB12	donor DNA with homology arms of 0.5 kb	1008
TB9/TB10	donor DNA with homology arms of 0.2 kb	407
TB72/CI21	Wild-type[1]	1226
	fcy1 mutant via HDR	Not amplified

[1]PC9 and 20b host strains.

Chapter 2

Functional analyses of *Pleurotus ostreatus pcc1* and *clp1* using CRISPR/Cas9

INTRODUCTION

Molecular mechanisms underlying the sexual development of tetrapolar heterothallic agaricomycetes are considered between controlled by *A* and *B* mating-type loci, which encode the homeodomain-type transcription factors, and pheromones and receptors, respectively (Raudaskoski, 2015). The mechanisms were investigated mainly in *Coprinopsis cinerea* and *Schizophyllum commune* using molecular genetics (Specht, 1995; Inada et al., 2001; Nakazawa et al., 2011b). Generally, dikaryotic cells are formed following the three main steps when two monokaryons with compatible alleles at both *A* and *B* loci are fused: (1) migration of nuclei across the colonies of two matings after hyphal fusion, (2) synchronous nuclear division and clamp cell formation, and (3) fusion of the clamp cell with the subterminal cell, causing dikaryon with clamp connection. In *C. cinerea*, (1) and (3) are regulated by the *B* locus, whereas (2) is regulated by *A* locus (Casselton et al., 1995; Kües, 2000). The resulting dikaryotic strains undergo a sexual cycle to form fruiting bodies under appropriate environmental conditions (Moore, 1998; Kamada et al., 2010).

Several genes essential or important for each of the aforementioned processes have been identified in *C. cinerea* and *S. commune* (Specht et al., 1995; Vaillancourt et al., 1997; Kamada, 2002; Kües and Navarro-González, 2015). For example, *pcc1* (pseudo clamp cell 1) gene mutations, which encode a putative high mobility group (HMG) transcription factor, result in pseudoclamp cell (clamp cell without clamp connection) formation in a *C. cinerea* monokaryon, suggesting that *pcc1* mutations activate the *A*-regulated pathways (Murata et al., 1998). The *pcc1-1* monokaryon 5337, exhibits a monokaryotic fruiting phenotype. Moreover, *pcc1* is upregulated in both *B*-on strains (strains in which the *B* pathway is constitutively active) and *A*-on strains (strains in which the *A* pathway is constitutively active) at the mRNA level in *C. cinerea* (Murata et al., 1998). Although the expression pattern seems to be inconsistent with the phenotypes of the *pcc1* mutant strain(s), these facts suggest that the mutations in *Ccpcc1* lead to overall activation of the *A*-regulated pathways. A mutation in the *clp1* gene [clampless 1; this gene is not homologous to *Saccharomyces cerevisiae CLP1*] was shown to result in block clamp cell formation in a *C. cinerea A43mut B43mut* strain 326 (the strain in which both *A*- and *B*-regulated pathways are partially activated; Inada et al., 2001). From these genetic studies, it was hypothesized that the *Cc*Clp1 protein might play an essential role in releasing the *A* pathway(s) from the *Cc*Pcc1 repression at a post-translational step, causing clamp

development in *C. cinerea* (Inada et al., 2001). These studies would help create monokaryotic fruiting strains of cultivated mushrooms with a gene replacement technique, providing useful and efficient molecular breeding without crosses. However, considering the diverse mechanisms of sexual development (Raudaskoski and Kothe, 2010; Kües et al., 2011; Raudaskoski, 2015), it remains unclear whether similar phenotypes are also observed in *pcc1* or *clp1* mutants of cultivated mushrooms.

Recently, CRISPR/Cas9 has been developed as an efficient gene targeting technique in some agaricomycetes, including *Pleurotus ostreatus*, *C. cinerea*, *S. commune*, and *Ganoderma lucidum* (Qin et al., 2017; Sugano et al., 2017; Vonk et al., 2019; Wang et al., 2020; Boontawon et al., 2021). However, it was rarely applied to functional analysis, unlike in classical gene targeting experiments using homologous recombination (de Jong et al., 2010; Nakazawa et al., 2011b, 2016a; Salame et al., 2012). This study analyzed the effects of *pcc1* and *clp1* mutations on sexual development in *P. ostreatus*, one of the most economically important cultivated mushrooms, to show an example of the application of CRISPR/Cas9 into functional analysis in cultivated mushrooms and investigate the diverse and conserved molecular mechanisms underlying sexual development in *Agaricomycetes* using CRISPR/Cas9.

MATERIALS AND METHODS

Strains, culture conditions, and genetic techniques of *P. ostreatus*

The *P. ostreatus* strains used are listed in Table 1. For routine cultures, yeast and malt extract with glucose (YMG) medium solidified with 2% (w/v) agar was used (Rao and Niederpruem, 1969). The cultures were maintained at 28°C under continuous darkness unless otherwise stated. Crosses and the production of fruiting bodies were performed as described by Nakazawa et al. (2016b). Genetic analysis using F_1 progenies was performed as described by Nakazawa et al. (2017). Hygromycin resistance transformation was performed using hygromycin resistance (*hph*) as described by Salame et al. (2012).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The total RNAs prepared from each *P. ostreatus* strain grown on YMG agar plate for 7 days were isolated by ISOGEN II RNA extraction reagent (Nippon Genetics, Tokyo, Japan). The obtained RNA samples were reverse transcribed using PrimeScript RT kit (Takara Bio, Shiga, Japan), followed by qRT-PCR. GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and Thermal Cycler Dice Real-Time System Lite (Takara Bio) were used. Quantification was carried out according to Pfaffl (2001). Primer pairs used for qRT-PCR and amplification

efficiencies for each pair are shown in Table S1 and S2.

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

Strain	Genotype/description	Source
PC9	*A2B1*/ 5-FC sensitive (Nakazawa and Honda, 2015)	CECT20311; Larraya et al. (1999)
PC15	*A1B2*	Larraya et al. (1999)
#64	*A64B64*	Nakazawa et al. (2017)
PC9×#64	*A2B1 A64B64*	This study
PC9×PC15	*A2B1 A1B2*	This study
PC15×#64	*A1B2 A64B64*	This study
pc1#1–2, 4–5, and 7–8	*A2B1*/ hygromycin-resistant strains obtained after introducing pCcPef3-126-*pcc1*sg1	This study
pc1#3	*A2B1 Popcc1-1*/ a hygromycin-resistant strain obtained after introducing pCcPef3-126-*pcc1*sg1	This study
pc1#6	*A2B1 Popcc1-2*/ a hygromycin-resistant strain obtained after introducing pCcPef3-126-*pcc1*sg1	This study
pc2#1–2, 4–5, and 7–9	*A2B1*/ hygromycin-resistant strains obtained after introducing pCcPef3-126-*pcc1*sg2	This study
pc2#3	*A2B1 Popcc1-3*/ a hygromycin-resistant strain obtained after introducing pCcPef3-126-*pcc1*sg2	This study
pc2#6	*A2B1 Popcc1-4*/ a hygromycin-resistant strain obtained after introducing pCcPef3-126-*pcc1*sg2	This study
pc2#3×PC15	*A2B1 A1B2 Popcc1-3 Popcc1*$^{+}$/ a cross of pc2-3×PC15	This study
pc2#6×#64	*A2B1 A64B64 Popcc1-4 Popcc1*$^{+}$/ a cross of pc2-6×#64	This study
pc2#3F$_1$#2, 6, 14, 16, 17, 21, 32, 42, 43 and 45	*Popcc1*$^{+}$/ a progeny of pc2#3×PC15	This study
pc2#3F$_1$#3, 4, 11, 15, 19, 41, 44 and 46	*Popcc1-3*/ a progeny of pc2#3×PC15	This study
pc2#6F$_1$#3, 8, 11, 13, 18, 26 and 27	*Popcc1*$^{+}$/ a progeny of pc2#6×#64	This study
pc2#6F$_1$#1 and 17	*A64B64 Popcc1-4*/ a progeny of pc2#6×#64	This study
pc2#6F$_1$#17×pc2#3F$_1$#6	*A64B64 AxBy Popcc1-4 Popcc1*$^{+}$/ a cross of pc2#6F$_1$#17 × pc2#3F$_1$#6	This study
cl1#6–7	*A2B1*/ hygromycin-resistant strains obtained after introducing pCcPef3-126-*clp1*sg1	This study
cl1#2	*A2B1 Poclp1-1*/ a hygromycin-resistant strain obtained after introducing pCcPef3-126-*clp1*sg1	This study
cl1#4	*A2B1 Poclp1-2*/ a hygromycin-resistant strain obtained after introducing pCcPef3-126-*clp1*sg1	This study
cl2#3 and 6	*A2B1*/ hygromycin-resistant strains obtained after introducing pCcPef3-126-*clp1*sg2	This study
cl1#2×PC15	*A2B1 A1B2 Poclp1-1 clp1*$^{+}$/ a cross of cl1#2×PC15	This study
cl1#4×#64	*A2B1 A64B64 Poclp1-2 clp1*$^{+}$/ a cross of cl1#4×#64	This study
cl1#2F$_1$#2, 4, 5, 8, 14, 15, 17–19, 22, and 25	*Poclp1*$^{+}$/ a progeny of cl1#2×PC15	This study
cl1#2F$_1$#1, 3, 6, 7, 9–13, 16, 20, 21, 23, 24, and 26–28	*Poclp1-1*/ a progeny of cl1#2×PC15	This study
cl1#4F$_1$#1, 3–5, 8–10, 12, 15, and 19	*Poclp1*$^{+}$/ a progeny of cl1#4×#64	This study
cl1#4F$_1$#2, 6, 7, 11, 13, 14, 16, and 18	*Poclp1-2*/ a progeny of cl1#4×#64	This study
cl1#4F$_1$#17	*A64B64 Poclp1-2*/ a progeny of cl1#4×#64	This study

Construction of plasmid for gene mutation using CRISPR/Cas9

The sgRNA sequences for introducing *Popcc1* and *Poclp1* mutations were designed using the Focas UI website (http://focas.ayanel.com). The following four sgRNA sequences

were used: *pcc1*sg1 (*Popcc1*; nucleotide positions 1242–1261 from the start codon), *pcc1*sg2 (*Popcc1*; positions 140–159), *clp1*sg1 (*Poclp1*; positions 791–810), and *clp1*sg2 (*Poclp1*; positions 439–458) (Table S1). The double-stranded DNA, which was prepared by annealing two DNA oligonucleotides [primers TB17/TB18 (*pcc1*sg1), TB19/TB20 (*pcc1*sg2), TB13/TB14 (*clp1*sg1), and TB15/TB16 (*clp1*sg2); Table S1], was separately inserted into the *Bsa*I site of the linearized pCcPef3-126 (Sugano et al., 2017) using the Golden Gate assembly (Engler et al., 2008, 2009). The correction of sgRNA insertion into plasmids was verified using Sanger sequencing. The resulting plasmids were named pCcPef3-126-*pcc1*sg1, pCcPef3-126-*pcc1*sg2, pCcPef3-126-*clp1*sg1, and pCcPef3-126-*clp1*sg2, respectively.

Genomic PCR experiments

For rapid genomic PCR experiments, fungal genomic DNA extraction and PCR techniques were conducted as described by Nakazawa et al. (2016b) and Boontawon et al. (2021). For genomic PCR to identify *Popcc1/Poclp1* mutations, the KOD FX Neo (Toyobo, Osaka, Japan) with a step-down cycle was used, followed by PCR fragment purification using the FastGene Gel/PCR Extraction Kit (Nippon Genetics) and then Sanger sequencing.

Fluorescent microscopy

Nuclei of fungi were visualized as described by Hua'an et al. (1991), with minor modifications. Briefly, a small amount of mycelium was grown for 3 to 5 days on a sterilized set of glass slides coated with a thin layer of the YMG medium on top of a filter paper moistened with distilled water inside a 9 cm plastic Petri dish. The slides were then dried and stained with PureBlu Hoechst 33342 nuclear staining dye (Bio-Rad, Hercules, CA, USA) in phosphate-buffered saline buffer [140 mM NaCl, 5 mM KH_2PO_4, and 1 mM $NaHCO_3$ (pH 7.4)]. The slides were observed under an Axio Scope.A1 fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany).

RESULTS

Identification of *P. ostreatus pcc1* and *clp1*

The candidate *P. ostreatus pcc1* and *clp1* were identified in the genome database of *P. ostreatus* PC9 (https://mycocosm.jgi.doe.gov/PleosPC9_1/PleosPC9_1.home.html) using BLASTp analysis against *C. cinerea* Pcc1 and Clp1 proteins [Murata et al., 1998, Inada et al., 2001; accession nos. AB007760 and AB034196, respectively, in the DNA Data Bank of Japan (http://getentry.ddbj.nig.ac.jp/top-e.html)]. Another *C. cinerea* protein annotated as Clp1 is

present in the NCBI (haccession no. XP_001833172); however, this is a homolog of *S. cerevisieae* Clp1. Protein ID 104201 in the *P. ostreatus* PC9 database exhibited the highest similarity of amino acid sequence with the *C. cinerea* Pcc1 (homology: 42.3%, *E*-value: 2.34 × 10^{-20}). Reciprocal BLASTp was performed against the amino acid sequence of the Protein ID to confirm its correspondence to the *C. cinerea* Pcc1 ortholog(s). Pcc1 (accession no. XP_001830477 in the National Center for Biotechnoilogy Information, NCBI) exhibited the highest similarity of amino acid sequences with Protein ID 104201 among predicted proteins from the database of *C. cinerea* okayama7#130 (NCBI: txid240176; *E*-value: 3 × 10^{-19}). These results indicated that the gene corresponding to the protein ID 104201 encodes the Pcc1 protein; therefore, it was designated as *P. ostreatus pcc1* (*Popcc1*).

As described by Scherer et al. (2006), two proteins exhibiting very high similarities are predicted in the databases of *P. ostreatus* and *C. cinerea* [Protein IDs 44201 and 47880 for *P. ostreatus*; XP_002911009/CC1G_15549 (*Cc*Clp1) and XP_001837806/CC1G_11451 for *C. cinerea*], which precluded us from determining which Protein ID is homologous to Clp1 by the homology search. Inada et al. (2001) showed that accumulation of *C. cinerea clp1* transcript is more abundant in the dikaryotic strain than in monokaryotic one, which allowed us to hypothesize that a *P. ostreatus* gene homologous to *clp1* also shows similar expression patterns. Therefore, accumulation of transcripts of the genes corresponding to the Protein IDs 44201 and 47880 were compared between monokaryotic and dikaryotic strains by qRT-PCR. The results (Fig. 1A and 1B) showed that transcript of 47880, but not that of 44201, accumulated much more abundantly in dikaryotic strains [#64×PC15, PC9×PC15, and PC9×#64] than monokaryotic strains (PC9 and #64), although the expression level in the monokaryotic strain PC15 was low. This suggested that 47880 might be homologous to Clp1 in *P. ostreatus*; therefore, the author designated the gene corresponding to the Protein ID 47880 as *P. ostreatus clp1*.

Popcc1 as well as *Poclp1* is upregulated in dikaryotic strains at the transcription level

To examine if *Popcc1* is also regulated by the *A*- and/or *B*-regulated pathways at the transcription level, *Popcc1* transcript accumulation was compared between monokaryotic and dikaryotic strains. As shown in Fig. 1C, *Popcc1* transcript, as well as *Poclp1* one, accumulated more abundantly in dikaryotic strains [#64×PC15, PC9×PC15, and PC9×#64] than monokaryotic strains (PC9, PC15, and #64). This result was consistent with the idea that the transcriptional expression of *Popcc1* (and *Poclp1*) is also under the control of the *A*- and/or *B*-regulated pathways, like in *C. cinerea* (Murata et al., 1998; Inada et al., 2001).

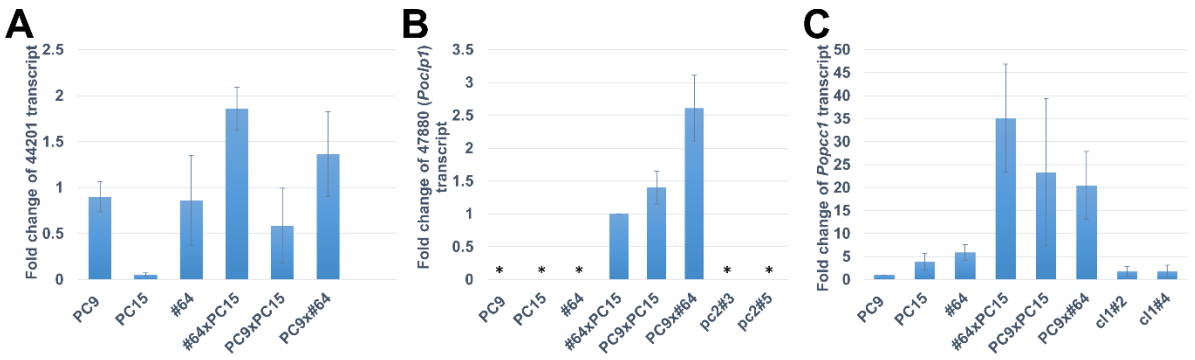

Fig. 1. Relative quantification of 44201, 47880 (*Poclp1*), and *Popcc1* transcripts in *P. ostreatus*. The bar charts indicate the 44201 (A), 47880 (*Poclp1*) (B), and *Popcc1* (C) expression level when normalized to the *β-tubulin* expression level compared among monokaryon (PC9, PC15, and #64), dikaryon (#64×PC15, PC9×PC15, and PC9×#64), and *Popcc1*/*Poclp1* mutant strains. Asterisks indicate no amplification/detection of *Poclp1*, of which the expression levels were considered as 0. Bars represent standard deviation (n = 3).

Introducing *Popcc1* or *Poclp1* mutations using plasmid introduction expressing Cas9 and sgRNA

Considering that the expression patterns of *Poclp1* and *Popcc1* are upregulated in dikaryotic strains, it was hypothesized that *Popcc1* and/or *Poclp1* might also be important in sexual development in *P. ostreatus*. To investigate their roles, CRISPR/Cas9-assisted gene mutagenesis by introducing a plasmid expressing Cas9 and sgRNA was used. The plasmids pCcPef3-126-*pcc1*sg1, pCcPef3-126-*pcc1*sg2, pCcPef3-126-*clp1*sg1, and pCcPef3-126-*clp1*sg2 were separately introduced into the *P. ostreatus* PC9 host strain (Table 1) to obtain hygromycin-resistant transformants. These plasmids express *Popcc1*- or *Poclp1*-targeting sgRNAs (*pcc1*sg1, *pcc1*sg2, *clp1*sg1, and *clp1*sg2, respectively) together with Cas9 and a hygromycin phosphotransferase (Hph) that confers resistance to hygromycin B. A total of eight, nine, nine, and 15 hygromycin-resistant strains were obtained by introducing pCcPef3-126-*pcc1*sg1, pCcPef3-126-*pcc1*sg2, pCcPef3-126-*clp1*sg1, and pCcPef3-126-*clp1*sg2, respectively.

Next, the *Popcc1/Poclp1* genotype (*Popcc1*$^{+}$/*Poclp*$^{+}$ or *Popcc1/Poclp1* mutant) was determined in all obtained hygromycin-resistant transformants by genomic PCR experiments. When CRISPR/Cas9 generates a double-strand break (DSB), an error-prone repair of DSB using the nonhomologous end-joining pathway generally causes small deletions and occasionally point mutation and large deletions, for example, several nucleotides insertion and a single nucleotide to several hundreds of nucleotides deletion at/around a target site (Wang et al., 2013; Wang et al., 2015). However, Boontawon et al. (2021) recently reported that the

introduced plasmid had been inserted at the target site of each sgRNA in the main population of *P. ostreatus fcy1* and *pyrG* mutant strains obtained from the pCcPef3-126-based CRISPR/Cas9; therefore, this study aimed to screen mutants with large deletion/insertion (indel) because the size of the PCR-amplified fragment can be easily distinguished between wild-type (WT) and mutant using conventional genomic PCR experiments; therefore, genomic fragments of the partial open reading frames (ORFs) of *Popcc1* (1.4 kb) and *Poclp1* (0.7 kb) containing the target sequences of sgRNA were first amplified using primer pairs TB23/TB24 and TB21/TB22, respectively (Fig. 2A), to screen *Popcc1* or *Poclp1* mutants. These fragments were anticipated to be amplified from PC9 but not from strains into which the large indel mutation had been introduced due to the loss of the hybridization site or required more extension time than the fragment amplified from the WT strain. The primer pair FY15/FY16, which amplifies a 0.4 kb genomic fragment from the ORF of *mer3* encoding an ATP-dependent DNA helicase (Protein ID 82484 in the JGI genome database), was also used as a positive control (Yamasaki et al., 2021).

The agarose gel electrophoresis results in some hygromycin-resistant strains are shown in Fig. 2. The positive control was amplified from all hygromycin-resistant strains used in this study (lanes 1–17 in Fig. 2B and lanes 1–6 in Fig. 2C). As shown in Fig. 2B and C, the expected fragments were amplified from the PC9 strain (lane WT) but not from three of eight hygromycin-resistant strains (lanes 3, 6, and 7 in Fig. 2B; strains pc1#3, 6, and 7, respectively), five of nine strains (lanes 11–15 in Fig. 2B; strains pc2#3–7, respectively), and two of four strains (lanes 1 and 2 in Fig. 2C; strains cl2#2 and 4, respectively) obtained after introducing plasmids pCcPef3-126-*pcc1*sg1, pCcPef3-126-*pcc1*sg2, and pCcPef3-126-*clp1*sg1, respectively, when the primer pairs TB23/TB24 and TB21/TB22 were used. These results suggested that these ten transformants could be *Poclp1*/*Popcc1* mutants with large indel mutations.

Identification of the CRISPR/Cas9 plasmid insertion at the target sites

To confirm the possibility of insertion of the introduced plasmid at the target site in some of the ten strains, genomic PCR experiments were performed as described by Boontawon et al. (2021). Four pairs of primers were designed to hybridize to the genomic region, which is located about 100 bp apart from the sgRNA target site in the first primer (primer TB23 or TB24 for *Popcc1*; primer TB21 or TB22 for *Poclp1*), and the *C. cinerea ß1-tub* promoter or terminator in the plasmids in the other primer (primer TN40 or TN46). The sets of primers TB23/TN40, TB23/TN46, TB24/TN40, and TB24/TN46 for *Popcc1* and TB21/TN40,

TB21/TN46, TB22/TN40, and TB22/TN46 for *Poclp1* were used for genomic PCR (Table S2). The PCR fragments about 7, 6, 8, and 2.6 kb were amplified from four hygromycin-resistant strains (namely pc1#3, pc1#6, pc2#3, and pc2#6, respectively) when the primer pairs TB24/TN40, TB24/TN40, TB23/TN40, and TB23/TN40 were used, respectively. In *Poclp1*, about 9 and 10 kb fragments were amplified from the two strains (namely cl1#2 and cl1#4, respectively) when the primer pairs TB21/TN46 and TB22/TN46 were used, respectively. These results suggested that the introduced plasmids had been inserted at the target sites at least in the six strains from which the fragment was amplified.

To verify the insertional mutation probabilities, the fragments were subjected to DNA sequencing using primers TB23 or TB24 for *Popcc1* and TB21 or TB22 for *Poclp1*. The results revealed the insertion of the introduced plasmids at the target sites of the four hygromycin-resistant transformants (strains pc1#3 and pc1#6 generated by introducing the pCcPef3-126-*pcc1*sg1 and strains pc2#3 and pc2#6 by pCcPef3-126-*pcc1*sg2; Fig. 3A). As for *Poclp1* mutations, the insertion of the introduced plasmid at the target site of *clp1*sg1 in the two strains, namely cl1#2 and cl1#4, is shown in Fig. 3B. The mutations in these strains, namely pc1#3, pc1#6, pc2#3, pc2#6, cl1#2, and cl1#4, were designated as *Popcc1-1*, *Popcc1-2*, *Popcc1-3*, *Popcc1-4*, *Poclp1-1*, and *Poclp1-2*, respectively (Table 1). These results suggested that the insertional mutations had been introduced into *Popcc1* or *Poclp1* in some of the hygromycin-resistant strains.

In Fig. S1A and B, the effects of *Popcc1-1*, *Popcc1-2*, *Popcc1-3*, *Popcc1-4*, *Poclp1-1*, and *Poclp1-2* on amino acid sequences of *Po*Pcc1 or *Po*Clp1 were illustrated. The analysis of the *Po*Pcc1 protein sequence alignment revealed that the premature stop codon was prospected to occur in strains pc1#3 (*Popcc1-1*), pc1#6 (*Popcc1-2*), pc2#3 (*Popcc1-3*), and pc2#6 (*Popcc1-4*) on the HMG box (at amino acid positions 402, 402, 54, and 58 from the start codon, respectively; Fig. S1A). In *Po*Clp1, the premature stop codon was predicted to occur in strains cl1#2 (*Poclp1-1*) and cl1#4 (*Poclp1-2*) (at positions 214 and 245, respectively; Fig. S1B). These results suggested that the insertional mutations cause the premature stop codon in *Popcc1* and *Poclp1* mutant strains.

Effects of *Poclp1* or *Popcc1* mutations on the transcriptional expression of these genes

In *C. cinerea, clp1* was inactivated in the *pcc1-1* mutant at the transcription level (Inada et al., 2001). To examine whether a similar effect would be observed in *P. ostreatus*, the author performed qRT-PCR. As shown in Fig. 1B, the *Poclp1* transcript was not detected in the *Popcc1* mutant strains pc2#3 (*Popcc1-3*) and pc2#5 like in monokaryotic strains, suggesting that

Poclp1 was not at least upregulated by *Popcc1* mutations. As for *Popcc1* transcription (Fig. 1C), it was a little bit increased (~1.8-fold) in strains cl1#2 (*Poclp1-1*) and cl1#4 (*Poclp1-2*) compared to the parental control strain PC9 but still less than the other monokaryon (PC15 and #64) and dikaryon strains. This result suggests that the expression level of *Popcc1* is not affected by *Poclp1* mutations at the transcription level.

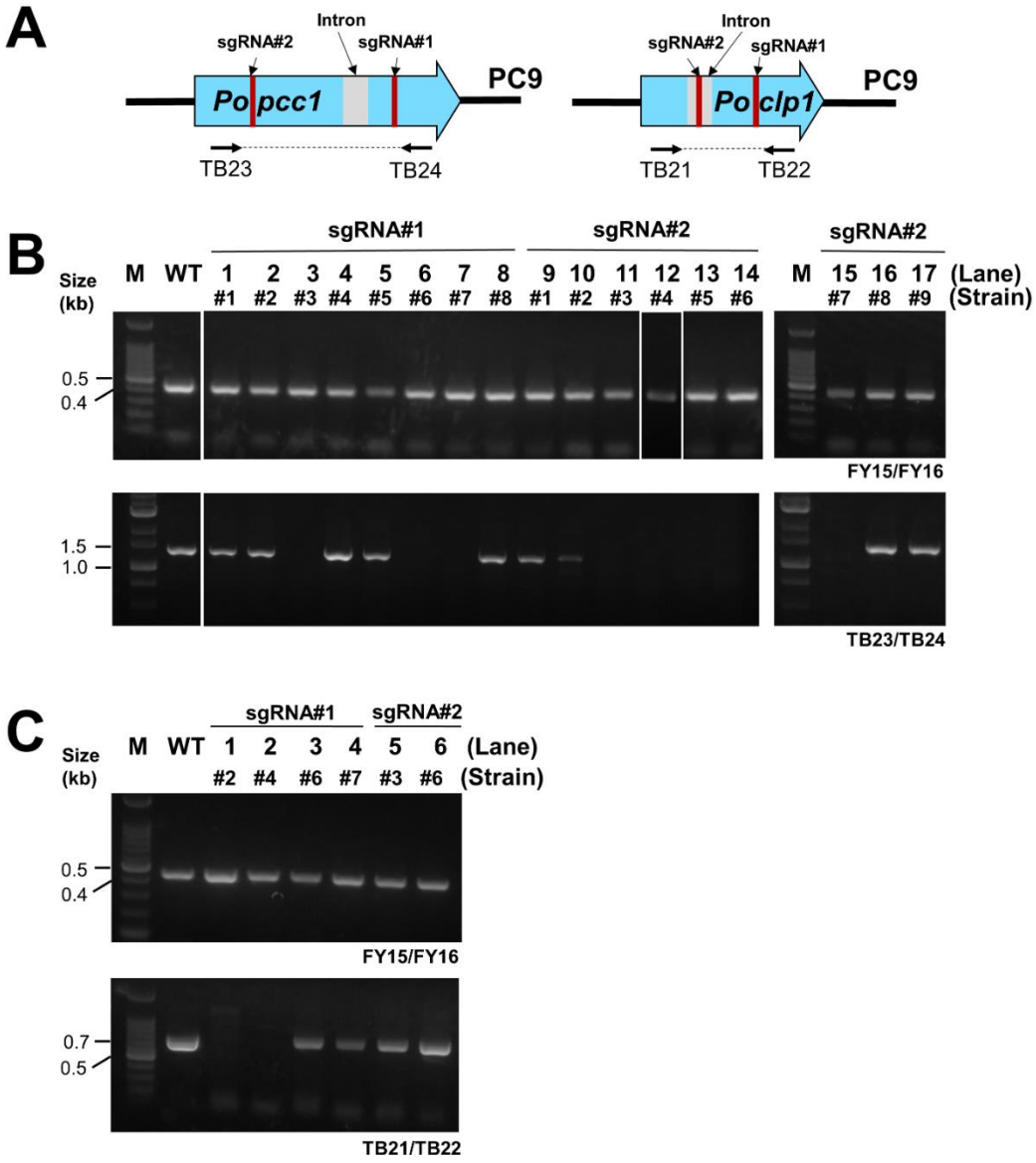

Fig. 2. Identification of *Popcc1* and *Poclp1* mutations in some strains using genomic PCR. (A) A schematic diagram of *Popcc1* and *Poclp1* loci in the PC9 host strain with sgRNA recognition sites. The dashed lines indicate the region amplified by genomic PCR. Black arrows display the primer pairs used for PCR experiments in B and C. Genomic PCR experiments identifying *Popcc1* (B) and *Poclp1* (C) mutations. Lane WT, parental strain PC9 used as a positive control; lanes 1–17 (B) and 1–6 (C), hygromycin-resistant strains; lane M, a 100 bp molecular weight marker (0.1–1.5 kb) or a 1 kb DNA ladder plus (0.1–10.0 kb). For more details regarding the estimated lengths of the PCR products amplified from the genome, see Table S2.

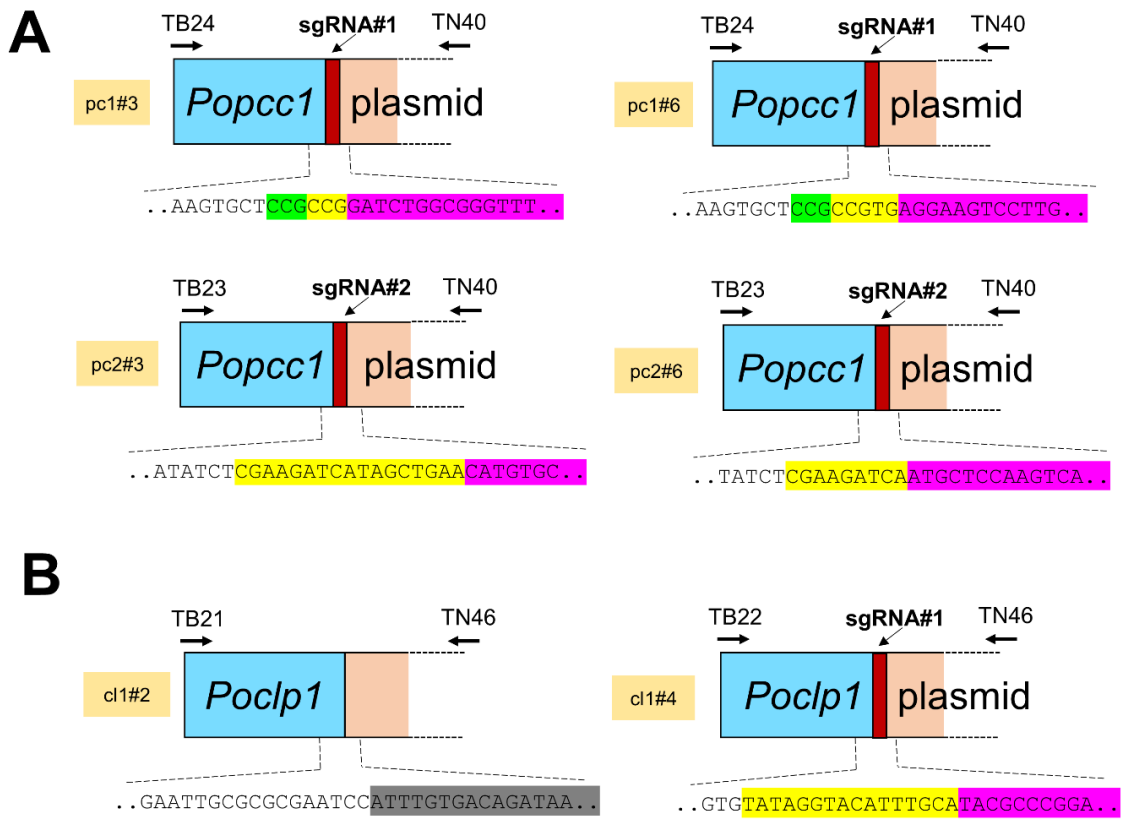

Fig. 3. Examination of small indel and insertional mutations in some strains. Identification of insertional mutations in some *Popcc1* (A) or *Poclp1* (B) mutants. Schematic diagrams showing how introduced plasmids had been inserted at the target sites of *Popcc1* or *Poclp1*. For highlights in the nucleotide sequence: yellow indicates sgRNA, green indicates a protospacer adjacent motif sequence, red indicates insertion, dashed lines indicate deletion, gray indicates the unknown sequence, and pink indicates the plasmid sequence.

Pseudoclamps were not observed in monokaryotic *P. ostreatus pcc1* mutants but they were after crossing

In *C. cinerea*, *Ccpcc1* mutations induce pseudoclamp cell formation in monokaryons (Murata et al., 1998; Murata and Kamada, 2009). To examine if a similar phenotype is observed in the monokaryotic *P. ostreatus pcc1* mutant, hyphal cells of strains pc1#3 (*Popcc1-1*), pc1#6 (*Popcc1-2*), pc2#3 (*Popcc1-3*), pc2#5, and pc2#6 (*Popcc1-4*) were observed under a microscope; however, no pseudoclamp cell was observed (Fig. S2). This suggested that *Popcc1* mutations do not induce clamp cell formation in the monokaryotic *P. ostreatus* strain PC9, which is not consistent with *C. cinerea*.

To further analyze the effects of *Popcc1* on sexual development, the effects of *Popcc1* mutations on clamp cell morphogenesis in dikaryon were also examined. First, F_1 progenies from a cross between the *Popcc1* mutants and WT strains were prepared for mating test analysis. The dikaryotic strains (namely pc2#3×PC15 and pc2#6×#64; Table 1) were obtained (Fig. 4A).

The basidiospores were harvested from a fruiting body to obtain/isolate F_1 progeny. To determine genotypes (*Popcc1*$^+$ or *Popcc1* mutation) of the resulting F_1 progenies, genomic PCR was conducted (Fig. S3A and B) as described in detail previously. The results revealed that ten *Popcc1*$^+$ (lanes 1, 4, 6, 8, 9, 11, 12, 14, 15, and 17 in Fig. S3A; strains pc2#3F_1#2, 6, 14, 16, 17, 21, 32, 42, 43, and 45, respectively) and eight *Popcc1-3* (lanes 2, 3, 5, 7, 10, 13, 16, and 18 in Fig. S3A; strains pc2#3F_1#3, 4, 11, 15, 19, 41, 44, and 46, respectively) monokaryotic strains were obtained from dikaryotic strains pc2#3×PC15 (Table 1). In the same way, nine *Popcc1*$^+$ (lanes 2–5 and 7–9 in Fig. S3B; strains pc2#6F_1#3, 8, 11, 13, 18, 26, and 27, respectively), and two *Popcc1-4* (lanes 1 and 6 in Fig. S3B; strains pc2#6F_1#1 and 17, respectively) one were obtained from dikaryotic strains pc2#6×#64 (Table 1). Then, the strain pc2#6F_1#17 (donor; *A64B64 Popcc1-4*; Table 1) was mated with the five F_1 progenies (*Popcc1*$^+$; strains pc2#3F_1#6, 17, 21, 32, and 42) from dikaryon pc2#3×PC15, as a recipient, which resulted in the formation of clamp cells with the connection within 5 days after mating, whereas those without the connection were observed after mating with the five F_1 progenies (*Popcc1-3*; strains pc2#3F_1#3, 4, 19, 44, and 46) as a recipient. To confirm the possibility of a pseudoclamp, a part of hyphae of pc2#6F_1#17×pc2#3F_1#4 was observed under a fluorescent microscope. As shown in Fig. 5, a fluorescent-stained nucleus is trapped in the unfused clamps in this dikaryotic strain, but not in the wild-type N001. This clearly indicates that they are indeed pseudoclamps. These results suggested that *pcc1* mutations do not induce *A*-regulated clamp cell formation in monokaryons but cause defects in *B*-regulated clamp connection in *P. ostreatus*.

Monokaryotic fruiting was observed in some *P. ostreatus pcc1* mutants

In *C. cinerea*, the monokaryotic *Ccpcc1* mutant could produce fruiting bodies after long cultivation (Murata et al., 1988). To examine whether monokaryotic *Popcc1* mutants also form fruiting bodies, monokaryotic *Popcc1* mutants [strains pc1#3 (*Popcc1-1*), pc1#6 (*Popcc1-2*), pc1#7, pc2#3 (*Popcc1-3*), pc2#5, and pc2#6F_1#17 (*Popcc1-4*); Table 1] were cultivated on sawdust-wheat bran medium. In this experiment, the pc2#6F_1#17×pc2#3F_1#6 [dikaryotic *Popcc1-4*×*Popcc1*$^+$ strain obtained from a cross between pc2#6F_1#17 and pc2#3F_1#6; Table 1] was also used as a positive control. As shown in Fig. 6, the *Popcc1-4*×*Popcc1*$^+$ dikaryotic strain showed a mature fruiting body (Fig. 6A), and three monokaryotic *Popcc1* mutants exhibited an irregular shape of the fruiting body (strains pc2#3 and pc2#6F_1#17; Fig. 6B) and a group of primordium (strain pc2#5; Fig. 6C); fruiting bodies were not formed from the remaining monokaryotic strains. This result suggested that fruiting bodies were induced in some

monokaryotic *Popcc1-3* and *Popcc1-4* mutants.

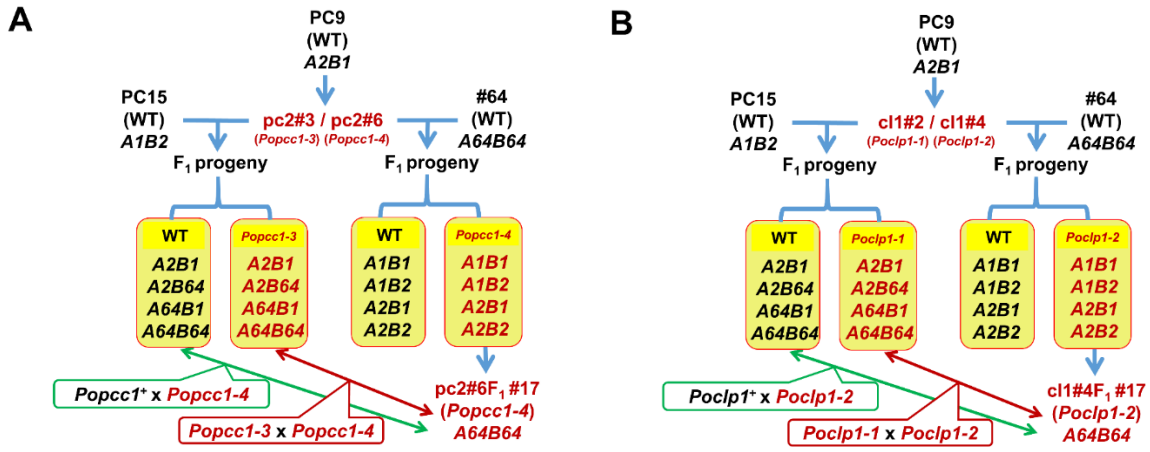

Fig. 4. Schematic diagrams showing how to perform mating tests using *Popcc1* (A) or *Poclp1* (B) mutants. The genotypes (WT or mutants) of the progenies were identified using genomic PCR (Fig. S3 and S5).

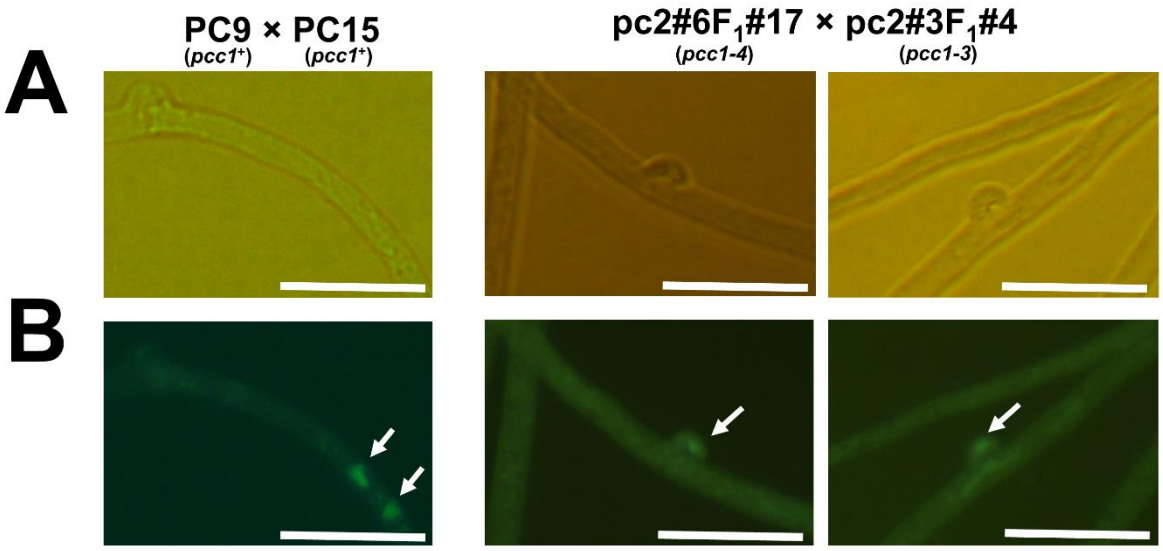

Fig. 5. Examination of clamp cell and pseudoclamp in a wild-type dikaryon [PC9×PC15 (N001)] and a dikaryotic *Popcc1* mutant [a cross between and pc2#6F$_1$#17 (*Popcc1-4*) and pc2#3F$_1$#4 (*Popcc1-3*)], respectively. Parts of hypha and clamp/pseudoclamp under light microscope (A) and fluorescence microscope (B). The arrow indicates nuclei. The bar represents 10 μm.

P. ostreatus clp1 mutations cause defects in dikaryosis

Considering that clamp formation was blocked with *Ccclp1-1* mutation in the *C. cinerea* strain 326 with the *A43mut B43mut* background (Inada et al., 2001), the following mating test was carried out using the *Poclp1* mutants to examine whether mutations cause defects in dikaryosis. First, the dikaryotic strains (namely cl1#2×PC15 and cl1#4×#64; Table

1) were obtained (Fig. 4B) from a cross between the *Poclp1* mutants and WT strains to obtain F_1 progenies used for the test. To determine genotypes (*Poclp1*$^+$ or *Poclp1* mutation) of the resulting F_1 progenies, genomic PCR was conducted (Fig. S4A and B). For the *Poclp1* genotype in the progenies, 11 *Poclp1*$^+$ (lanes 2, 4, 5, 8, 14, 15, 17–19, 22, and 25 in Fig. S4A; strains cl1#2F_1#2, 4, 5, 8, 14, 15, 17–19, 22, and 25, respectively) and 17 *Poclp1-1* (lanes 1, 3, 6, 7, 9–13, 16, 20, 21, 23, 24, and 26–28 in Fig. S4A; strains cl1#2F_1#1, 3, 6, 7, 9–13, 16, 20, 21, 23, 24, and 26–28, respectively) monokaryotic strains were obtained from dikaryotic strains cl1#2×PC15 (Table 1). In the same way, ten *Poclp1*$^+$ (lanes 1, 3–5, 8–10, 12, 15, and 19 in Fig. S4B; strains cl1#4F_1#1, 3–5, 8–10, 12, 15, and 19, respectively) and nine *Poclp1-2* (lanes 2, 6, 7, 11, 13, 14, and 16–18 in Fig. S4B; strains cl1#4F_1#2, 6, 7, 11, 13, 14, and 16–18, respectively) were obtained from dikaryotic strains cl1#4×#64 (Table 1).

To determine the effects of *Poclp1* on dikaryosis, the following genetic analysis was performed. The strain cl1#4F_1#17 (*A64B64 Poclp1-2*; Table 1) was used as a donor. Next, the strain cl1#4F_1#17 was mated with eight F_1 progenies (*Poclp1*$^+$; strains cl1#2F_1#2, 4, 5, 8, 14, 18, 22, and 25) from the dikaryon cl1#2×PC15, as a recipient, which resulted in clamp connection within 5 days after mating. In contrast, a clamp cell was not observed after mating with seven F_1 progenies (*Poclp1-1*; strains cl1#2F_1#13, 20, 21, 24, and 26–28) as a recipient. This result suggested that *Poclp1* mutations cause defects in dikaryosis.

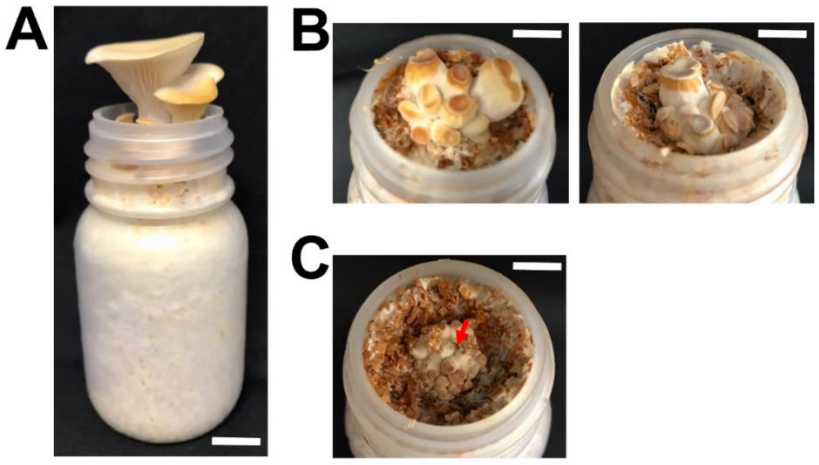

Fig. 6. Monokaryotic fruiting in strains pc2#3, pc2#6F_1#17, and pc2#5. An intact fruiting body from a dikaryotic strain pc2#6F_1#17×pc2-3F_1#6 as a positive control (A). A monokaryotic fruiting body formed on the mycelium of strains pc2#3 and pc2#6F_1#17, respectively (B), and pc2#5 (C). A red arrow indicates a group of primordia. Bar, 1 cm.

DISCUSSION

This study examined the effects of *Popcc1* or *Poclp1* mutations on the sexual development in *P. ostreatus* using CRISPR/Cas9 to show an example for the application. The *Popcc1* or *Poclp1* mutants with large insertional mutations were selected for phenotypic and genetic analyses. Although it may be easier to clarify or reveal small indel mutations in a target gene by Sanger sequencing, large ones are useful for genetic analysis in basic studies because the genotype (mutant or WT) can be easily determined by conventional genomic PCR experiments as performed in this study. Therefore, although it is important to develop the marker-free CRISPR system to introduce the small mutations for the molecular breeding of nongenetically modified strains of *P. ostreatus*, the current system that can generate mutants with insertional mutations would be convenient for molecular genetic studies.

This study showed that the transcriptional expression of *P. ostreatus pcc1* and *clp1* is higher in dikaryotic strains than in monokaryotic ones; however, it remains unclear whether they are under the control of the *A*- and/or *B*-regulated pathways in *P. ostreatus*. Kamada (2002) proposed that *clp1* could be directly regulated by the *A*-protein heterodimer (HD1 and HD2) as an *hsg*-like element ($GATGX_{11}ACA$) is located 219- to 201-bp upstream of the start codon of *Ccclp1*, although this remains to be investigated. However, the *hsg*-like element was not found in the 5′ upstream region of *Poclp1* despite the fact that transcriptional expression of *Poclp1* was higher in the dikaryotic strains than in the monokaryotic strains. This suggests that *clp1* may not be regulated by the heterodimer directly at least in *P. ostreatus*. This could reflect differences in the regulation mechanisms of the *A*-regulated pathways between *C. cinerea* and *P. ostreatus*.

Furthermore, although both *P. ostreatus pcc1* and *clp1* single-gene mutants exhibited the mutant phenotypes probably associated with sexual development, it could not be concluded that they were indeed caused by the defects in *A*- and/or *B*-regulated pathways as the role of the two mating-type loci in each process of sexual development has not been clarified/elucidated in *P. ostreatus*. Although the roles were extensively investigated in *C. cinerea* and *S. commune* (Kamada, 2002; Raudaskoski, 2015), the possibility that the *A*- and/or *B*-regulated pathways in *P. ostreatus* (and other agaricomycetes) are not identical to those in the model species cannot be elucidated; therefore, *A*-on or *B*-on *P. ostreatus* strains should be created to investigate the role of the *A*- and *B*-regulated pathways. In *C. cinerea*, transcriptome analyses using *A43mut* and/or *B43mut* strains were reported (Stajich et al., 2010); however, analyses downstream of the *A*- and *B*-regulated pathways as well as of Pcc1 remains unclear. More extensive comparative transcriptome analyses between *C. cinerea* and *P. ostreatus A*-on,

B-on, *clp1* or *pcc1* mutants would elucidate the conserved and diverse mechanisms/pathways in the future.

Unlike *C. cinerea*, *Popcc1* mutations did not induce clamp cell formation in the strain PC9. In *C. cinerea*, various mutations, including *Ccpcc1-2*, *Ccpcc1-3*, and *Ccpcc1-4* that lead to the synthesis of a short peptide lacking the HGM box (Murata and Kamada, 2009), induce clamp cell formation; in this study, clamp cell connection was impaired when *Popcc1-3* monokaryon was crossed with *Popcc1-4* one: the effects of the both *Popcc1* mutations on amino acid sequence of *Po*Pcc1 are similar to those of the aforementioned three *Ccpcc1* ones. Assuming that the roles of the *A*- and *B*-regulated pathways are conserved between *C. cinerea* and *P. ostreatus*, these results suggest that *P. ostreatus pcc1* mutations cause defects in some of the *B*-regulated pathways, and do not activate all the *A*-regulated pathways required for clamp cell formation without mating (Fig. 7). If this is correct, some of the *B*-regulated pathways are possibly impaired in *C. cinerea pcc1* mutants, which could explain the inconsistency between the phenotype and transcriptional expression pattern observed in *C. cinerea* (Murata et al., 1998). Although it remains unclear whether another *B*-regulated process, nuclear exchange after hyphal fusion, is also impaired in *P. ostreatus pcc1* mutants, this could reflect the diversity in pathways downstream of Pcc1 among *Agaricomycetes.*

Like in *C. cinerea*, fruiting development was induced to some degree in monokaryotic *P. ostreatus pcc1-3* and *pcc1-4* mutants but not the other mutants. This result suggested that *pcc1* mutants can be applied to efficient molecular breeding of cultivated mushrooms; however, monokaryotic fruiting induction was incomplete (the fruiting bodies did not mature normally) in both *C. cinerea* and *P. ostreatus* possibly because this suggested the mutations may not activate all the pathways required for normal fruiting as the *A* and/or *B* pathways may not be activated completely, which should be investigated by the comparative transcriptome analyses using the monokaryotic *pcc1* mutants and WT dikaryotic strains to generate monokaryotic strains that form fruiting bodies normally. No *Ccpcc1* mutation leading to the synthesis of the mutant Pcc1 that lacks the short C-terminal region, like the *Popcc1-1* and *Popcc1-2* (Fig. S1), was reported to induce pseudoclamp formation and fruiting in the monokaryotic strain (Murata et al., 2009). Therefore, these results suggest that the *P. ostreatus pcc1-1* and *pcc1-2* mutations may not impair the function(s) of Pcc1 completely.

As for *Poclp1* mutations, it was shown that they cause defects in dikaryosis. Likely, *Poclp1* is also essential for clamp cell formation, which is possibly under the control of the *A*-regulated pathways, like in *C. cinerea* (Inada et al., 2001); however, considering that Clp1 was suggested to affect the pheromone siglanling pathways as well as the homeodomain proteins-

dependent ones in *Ustilago maydis* (Scherer et al., 2006; Heimel et al., 2010), the possibility that process (es) of hyphal fusion or nuclear exchange was also impaired in *P. ostreatus clp1* mutants cannot be excluded. To clarify this issue, the effects of *Poclp1* mutation(s) on clamp cell formation in a homokaryotic fruiting strain should be analyzed. It should also be examined whether overexpressing *Poclp1* in monokaryotic strains, such as PC9, induces clamp cell (pseudoclamp) formation.

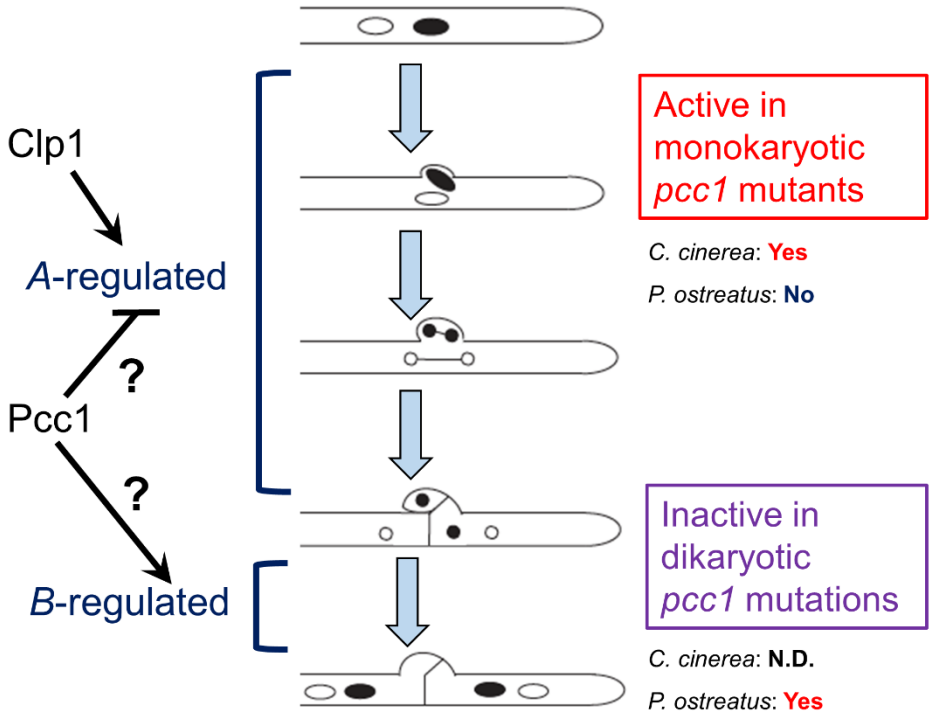

Fig. 7. A synthesis of knowledge on the effects of the *pcc1* mutations on clamp cell development in *C. cinerea* and *P. ostreatus* from this study and Murata et al. (1998; 2009). The diagrams of hyphal cells are from Nakazawa et al. (2011a). "N.D." indicates "not determined".

SUMMARY

In summary, the findings provided a clue with respect to conserved and diverse mechanisms underlying sexual development in *Agaricomycetes* (at least between *C. cinerea* and *P. ostreatus*) by functional analyses of *P. ostreatus clp1* and *pcc1* using CRISPR/Cas9. However, unlike in gene targeting experiments using homologous recombination, the possibility that ectopic integration of the plasmids used for introducing mutations by CRISPR/Cas9 affects the phenotypes cannot be generally excluded. Although genetic/linkage analyses performed in this study excluded this possibility, the methods to introduce CRISPR/Cas9-assisted mutations without ectopic integration should be developed to make them more useful tools for molecular genetic studies in *Agaricomycetes*.

SUPPLEMENTARY DATA

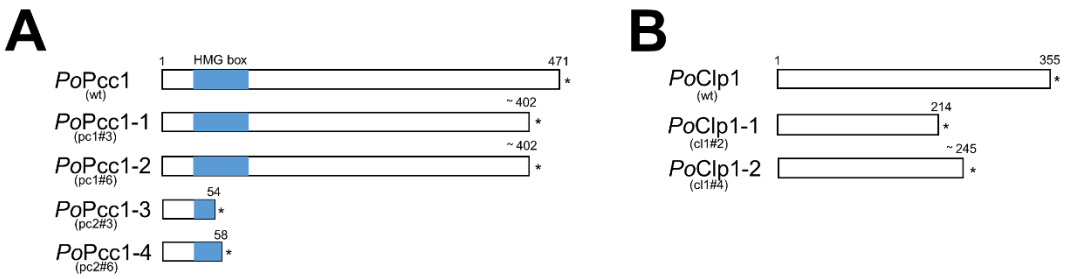

Fig. S1. The putative structures of *Po*Pcc1 and *Po*Clp1 based on BLAST search. The effect of *Popcc1* (*Popcc1-1*, *Popcc1-2*, *Popcc1-3*, *Popcc1-4*; A) and *Poclp1* (*Poclp1-1*, *Poclp1-2*; B) mutations on protein function are indicated with asterisks. Blue shades indicate the predicted HMG box.

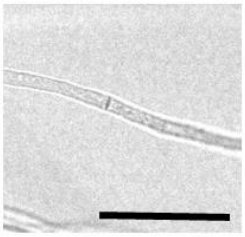

Fig. S2. Examination of pseudoclamp in monokaryotic *Popcc1* mutant strain pc2#3. An image shows a part of hypha of strain pc2#3 (*Popcc1-3*) under light microscope. The bar represents 5 μm.

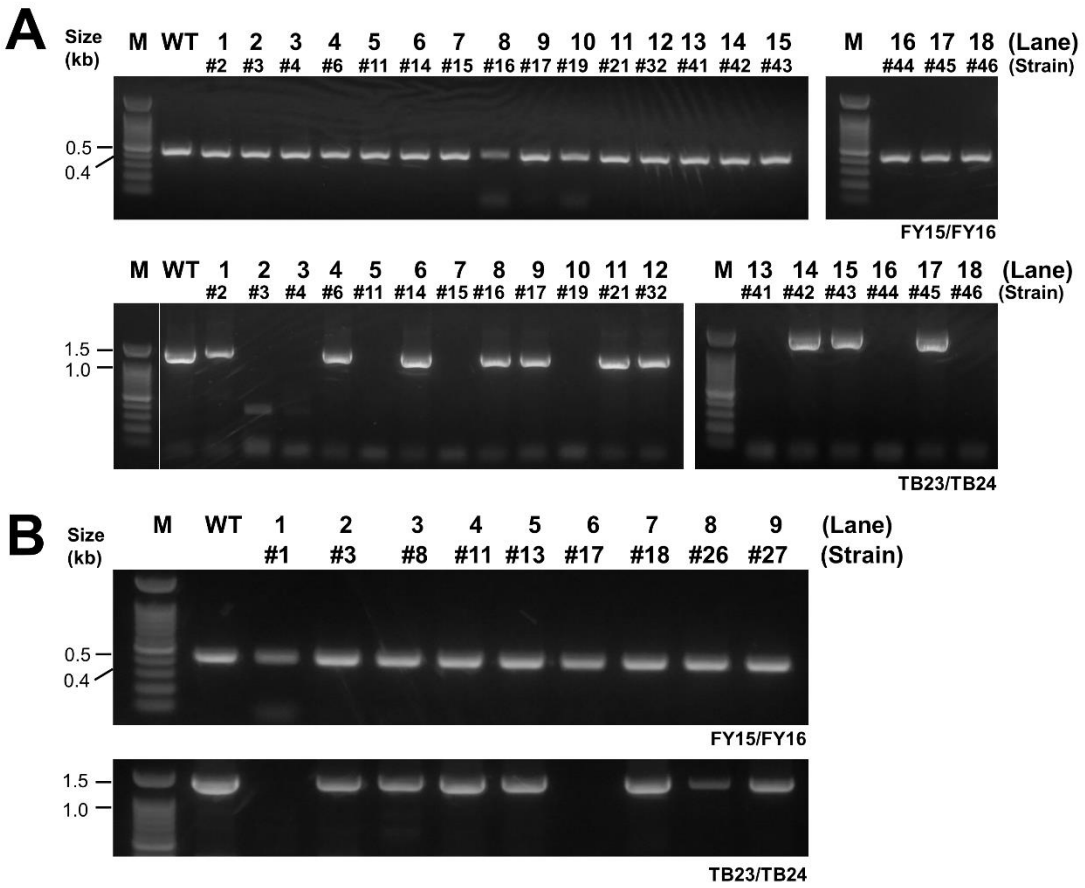

Fig. S3. Genomic PCR experiments examining *Popcc1* mutation. The agarose gel electrophoresis of the F_1 progeny from dikaryon strains pc2#3×PC15 (A) and pc2#6×#64 (B). Lane WT, the parental strain PC9 as a positive control; Lanes 1–18 (A) and 1–9 (B), F_1 progenies; Lane M, a 100-bp molecular weight marker (0.1–1.5 kb). For more details regarding the estimated lengths of the PCR products amplified from the genome, please see Table S2.

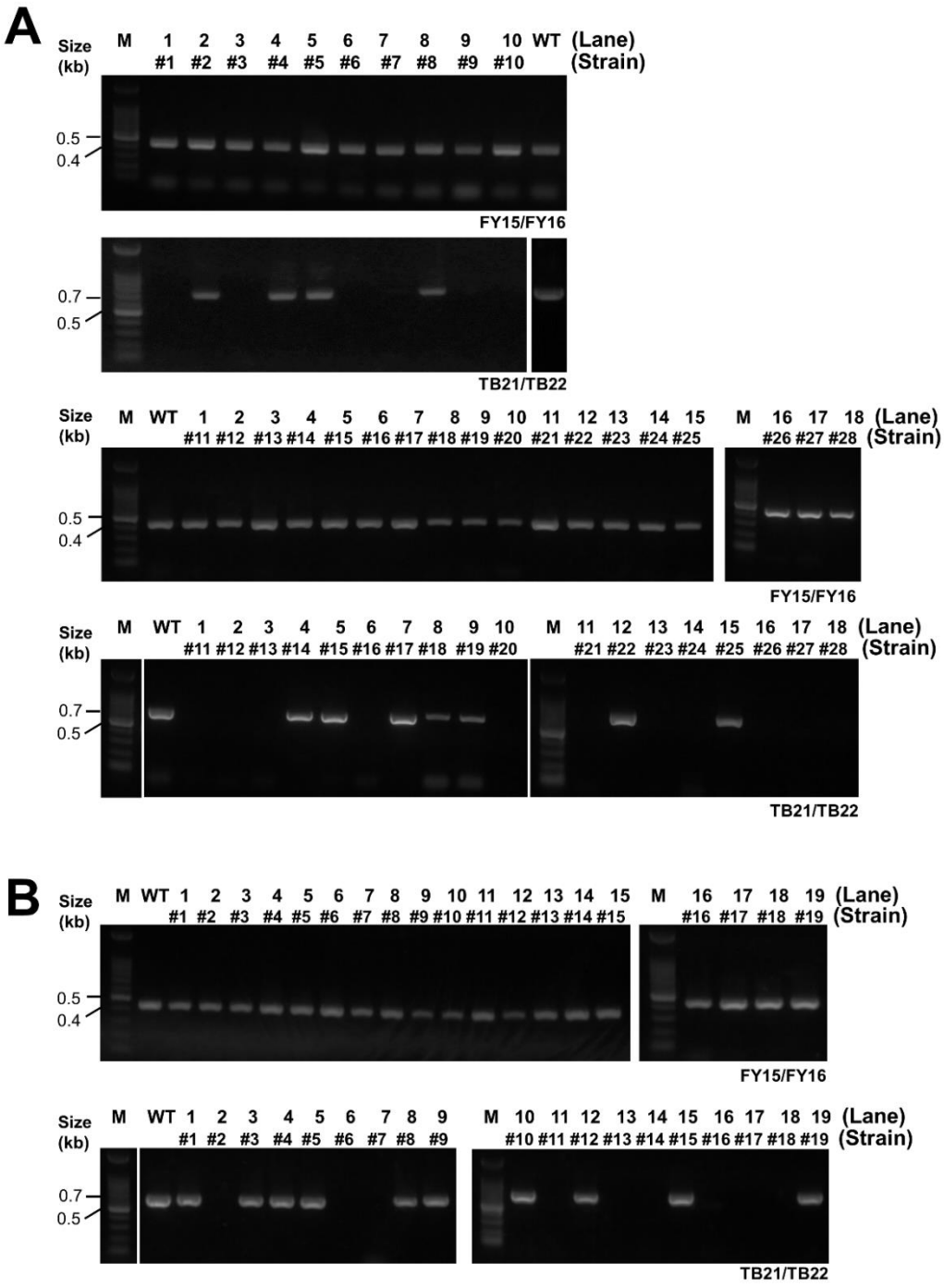

Fig. S4. Genomic PCR experiments examining *Poclp1* mutation. The agarose gel electrophoresis of the F_1 progeny from dikaryotic strains cl1#2×PC15 (A) and cl1#4×#64 (B). Lane WT, the parental strain PC9 as a positive control; Lanes 1–18 (A) and 1–9 (B), F_1 progenies; Lane M, a 100-bp molecular weight marker (0.1–1.5 kb). For more details regarding the estimated lengths of the PCR products amplified from the genome, please see Table S2.

Table S1. Primer pairs used in this study

Primer	**Sequence (5' to 3')**
FY15	AACCCCAAGTTCTATGCGTTG
FY16	ACTTACTCTTCTGCAGCCGAC
TB13	GATTGTATAGGTACATTTGCAGAA
TB14	AAACTTCTGCAAATGTACCTATAC
TB15	GATTGTTGAAGGTCGAGTGAGACAA
TB16	AAACTTGTCTCACTCGACCTTCAAC
TB17	GATTGTTTCGCGTCATATAACACGG
TB18	AAACCCGTGTTATATGACGCGAAAC
TB19	GATTGCGAAGATCATAGCTGAACGG
TB20	AAACCCGTTCAGCTATGATCTTCGC
TB21	CTACACCATCTCCTACCACCGTC
TB22	CAAGGACAGCCTGAGTGTGCTC
TB23	CCTCCACGACCTTCAAACCGC
TB24	GGCACATAGGACGAAGACGACG
TB84	CGCTGCTTCACCAGAGTTGGC
TB85	CGAATTGGGATGGAGAGAGATGATGG
TN40	ACCCTTTCCCCCAAAATTTGGAAGC
TN46	AAACGGCTTCACGGGCAGCC
TN97	TCCTAGACGACGAGTAGAGACAGGC
TN98	CATTCTGCATGGAAAAGAAGTTAGTCGC
TN201	TCCCGCCTACCGATTCTGAG
TN202	TCCGCCGTGTTATATGACGC
TN203	TACACCATCTCCTACCACCGTCCC
TN204	TCTGCTGGCGCTTCGTGTATAGG

Table S2. Estimated lengths of the PCR fragments that were amplified from each strain.

Primer set	Strain/ Function	Estimated length (kb)
TN97/TN98	qRT-PCR of *β-tubulin* gene (primer efficiency: 96.3; Salame et al., 2012)	0.1
TN201/TN202	qRT-PCR of *Popcc1* gene (primer efficiency: 122.8)	0.1
TN203/TN204	qRT-PCR of 47880 (*Poclp1*) gene (primer efficiency: 111.2)	0.5
TB84/TB85	qRT-PCR of 44201 (primer efficiency: 77.8)	0.1
TB13/TB14	*Poclp1* sgRNA no.1	-
TB15/TB16	*Poclp1* sgRNA no.2	-
TB17/TB18	*Popcc1* sgRNA no.1	-
TB19/TB20	*Popcc1* sgRNA no.2	-
TB21/TB22	verification of *Poclp1* mutation	0.7
TB23/TB24	verification of *Popcc1* mutation	1.4
FY15/FY16	*mer3* gene in *P. ostreatus*	0.4
TB21/TN40	wild-type	Not amplified
	Poclp1 mutant	Large indels of nucleotide, or not amplified
TB21/TN46	wild-type	Not amplified
	Poclp1 mutant	Large indels of nucleotide, or not amplified
TB22/TN40	wild-type	Not amplified
	Poclp1 mutant	Large indels of nucleotide, or not amplified
TB22/TN46	wild-type	Not amplified
	Poclp1 mutant	Large indels of nucleotide, or not amplified
TB23/TN40	wild-type	Not amplified
	Popcc1 mutant	Large indels of nucleotide, or not amplified
TB23/TN46	wild-type	Not amplified
	Popcc1 mutant	Large indels of nucleotide, or not amplified
TB24/TN40	wild-type	Not amplified
	Popcc1 mutant	Large indels of nucleotide, or not amplified
TB24/TN46	wild-type	Not amplified
	Popcc1 mutant	Large indels of nucleotide, or not amplified

Chapter 3

Advanced method in gene targeting using pre-assembled Cas9 ribonucleoprotein and split-marker in non-*ku80 Pleurotus ostreatus* strain

INTRODUCTION

Several of the economically important mushroom-forming fungal species belong to the class *Agaricomycetes*, such as *Agaricus bisporus* (button mushroom), *Pleurotus ostreatus* (oyster mushroom), and *Lentinula edodes* (shiitake mushroom). Extensive breeding of cultivated mushroom species has generated strains with traits to improve cultivation and food traits. However, classical breeding is laborious and takes a long time because of the several crossing steps involved (Sonnenberg et al., 2008). Moreover, a tetrapolar mating system, which is present in most cultivated mushrooms, makes crossing and breeding more complicated (Raudaskoski and Kothe 2010; Kües et al., 2011). Therefore, new methodologies for more efficient and straightforward molecular breeding are necessary.

Various methodologies have been used for molecular breeding. For example, an efficient gene targeting using homologous recombination was applied to generate sporeless *P. ostreatus* strains by disrupting *msh4* or *mer3* in the non-homologous end joining (NHEJ)-deficient strain 20b (Yamasaki et al., 2021; Salame et al., 2012). However, the strains generated using this technique are classified as genetically modified organisms (GMO) because of the introduction of a foreign DNA sequence. This presents a challenge since GM crops and their products are completely prohibited in some countries (Germany and France), while they are permitted in limited numbers in the Czech Republic and Spain (Mahaffey et al., 2016). Therefore, to generate mushroom strains that can be more widely accepted by societies worldwide, new techniques for the molecular breeding of non-GM mushrooms are needed.

CRISPR/Cas9, which was first discovered as an adaptive immune system in bacteria and archaea (Ishino et al., 1987; Jinek et al., 2012), has been applied as a gene-targeting tool for genome editing. It is composed of two main parts: Cas9 endonuclease for generating double-stranded DNA breaks (DSBs) and a single guide RNA (sgRNA) to guide Cas9 to the target site on the chromosome. In some agaricomycetes, such as *P. ostreatus*, *Coprinopsis cinerea*, and *Ganoderma lucidum*, CRISPR/Cas9 has been introduced as an efficient gene targeting method via plasmid introduction (Qin et al., 2017; Sugano et al., 2017; Wang et al., 2020; Boontawon et al., 2021). A pre-assembled ribonucleoprotein complex of Cas9 protein with *in vitro* transcribed sgRNAs (Cas9 RNP) is an alternative way to perform gene

mutation/disruption in various organisms without the possibility of plasmid integration. This technique can be useful for molecular breeding applications, as it can be used for breeding using gene targeting without the introduction of foreign DNA sequence(s). However, to date, in *Agaricomycetes*, genome editing using Cas9 RNP has been reported only in *Schizophyllum commune* (Jan Vonk et al., 2019), a model species for molecular genetics studies of sexual development (Raudaskoski et al., 2015). In this study, the author demonstrates, for the first time, an efficient gene mutagenesis in a monokaryotic wild-type strain of the popular cultivated mushroom *P. ostreatus* by introducing pre-assembled Cas9 RNP.

NHEJ-deficient strains are useful for gene-targeting experiments, but they were also partially defective in meiotic processes and fruit maturation in *C. cinerea*, as reported in previous studies (Namekawa et al., 2003; Nakazawa et al., 2011b). In *Neurospora crassa*, NHEJ-deficient strains exhibit higher sensitivity to mutagens such as ethyl methanesulfonate, which induces random point mutations in DNA and RNA by guanine alkylation, when compared to that by the wild-type strain (Ninomiya et al., 2004; Ishibashi et al., 2006). This may be the result of a lower activity of the NHEJ-mediated DNA repair system. Such deficiency would preclude us from determining the precise effects of targeted disruption of the gene of interest in some cases. Furthermore, in *Agaricomycetes*, efficient gene targeting techniques were available only in *C. cinerea*, *S. commune*, *P. ostreatus*, and *G. lucidum*. Although genetic transformation has been reported in various agaricomycetes, such as the ectomycorrhizal symbiont *Laccaria bicolor* and the white-rot fungus *Phanerochaete chrysosporium* (Alic et al., 1990; Kemppainen et al., 2005), it is challenging to conduct gene targeting experiments in them, especially since NHEJ-deficient strains have not been generated. To solve this problem, the author demonstrated another gene targeting method, a split-marker along with Cas9 RNP, that can be performed in non-model agaricomycetes, even if NHEJ-deficient recipient strain is not available.

MATERIALS AND METHODS

Strains, culture conditions and genetic techniques

Pleurotus ostreatus monokaryotic wild-type strain PC9 (Spanish Type Culture Collection accession number CECT20311) and the other 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 was used to maintain fungal cultures. The final concentration of 0.18 mM uracil and 20 mM uridine were added to the YMG medium (YMGUU) to grow uracil and uridine auxotrophic strains. When necessary, 0.1% (w/v) 5-fluoroorotic acid (5-FOA), 0.18

mM uracil, and 20 mM uridine were added to the medium.

The transformation of *P. ostreatus* strain using the hygromycin resistance gene (*hph*) or orotidine 5'-phosphate decarboxylase-encoding gene (*pyrG*) was performed using protoplasts prepared from mycelial cells as described by Salame et al. (2012), with the exception that heparin and single-strand lambda phage DNA were not used.

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

Strain	Genotype/description	Source
PC9	*A2B1*/5-FC and 5-FOA-sensitive (Nakazawa et al., 2016b)	CECT20311
py#1	*A2B1 pyrG-8*/a 5-FOA-resistant strain obtained after introducing Cas9/*pyrG*sg1 RNP	This study
py#2	*A2B1 pyrG-9*/a 5-FOA-resistant strain obtained after introducing Cas9/*pyrG*sg1 RNP	This study
py#3	*A2B1 pyrG-10*/a 5-FOA-resistant strain obtained after introducing Cas9/*pyrG*sg1 RNP	This study
py#4–5	*A2B1 pyrG-11*/5-FOA-resistant strains obtained after introducing Cas9/*pyrG*sg1 RNP	This study
sr#1–3, 5–10 and 14–16	*A2B1 pyrG*::*Hyg*^{R} /5-FOA-resistant strains obtained after introducing Cas9/*pyrG*sg1 RNP and split-marker donor DNA templates	This study
sr#4 and 11–13	*A2B1* /5-FOA-resistant strains obtained after introducing Cas9/*pyrG*sg1 RNP and split-marker donor DNA templates	This study

Design and synthesis of gene-specific sgRNAs for gene disruption

The sgRNA sequence for targeting *pyrG* at nucleotide position 521–540 from the start codon (Nakazawa et al., 2016b) was obtained from our previous study (Boontawon et al., 2021; Table S1). The DNA template for *in vitro* synthesis of sgRNA was designed based on the protocol of the *CUGA*7 sgRNA synthesis kit (protocol No. 314-08691; Nippon Genetics, Tokyo, Japan) with minor modifications to the sgRNA scaffold to increase the efficiency of gene disruption (Dang et al., 2015; Table S1). The set of synthesised oligonucleotides containing the T7 promoter, *pyrG* sgRNA sequence, and sgRNA scaffold (primers TB68, TB69, and TB70; Table S2) were assembled to construct the *pyrG* sgRNA DNA template using KOD FX Neo (Toyobo, Osaka, Japan). PCR products from the previous reactions were purified from an agarose gel using a FastGene Gel/PCR Extraction Kit (Nippon Genetics) and used as templates for mass amplification using TB71/CI78. sgRNA was synthesised and purified using the same kit. The concentration of the DNA template and the sgRNA were measured using Qubit 1× dsDNA and RNA HS assay kits, respectively (Thermo Fisher Scientific, Waltham, MA, USA).

***In vitro* Cas9 digestion assay**

For the *in vitro* cleavage assay, 0.5 μg of Cas9 protein (Nippon Genetics) and 250 ng of sgRNA (around 1:2 molar ratio) were mixed to a final volume of 5 μl and assembled at 37 °C for 5 min. After assembly of the Cas9 RNP, 400 ng of PCR-amplified fragment containing a target site for the sgRNA and 1× H buffer (1 M NaCl, 500 mM Tris-HCl pH 7.9, 100 mM

$MgCl_2$, 10 mM DTT) were added. The resulting sample (20 µl) was incubated at 37 °C for 50 min. Cas9 RNP was released by the addition of 10% (w/v) SDS to a final concentration of ~1% (w/v) and incubated at 65 °C for 5 min. Finally, 15 µl of the reaction was run on an agarose gel to verify DNA digestion.

Design of the repair templates for homologous recombination

For precise gene replacement using split-marker recombination in the *pyrG* gene, right and left donor DNA templates containing half of the hygromycin resistance gene (*hph* cassette), with homology arms of 1 kb, were constructed using overlap extension PCR. Approximately 1 kb genomic fragments containing 5′-upstream and 3′-downstream sequences were amplified using TB62/TB63 and TB64/TB65, respectively. A 1.9 kb hygromycin-resistance cassette was amplified from the plasmid pCcPef3-126 (Sugano et al., 2017) using TB60/TB61. These fragments were fused by TB62/TB65 to obtain an intact donor DNA (3.9 kb in total) followed by amplification with TB62/TB66 and TB65/TB67 resulting in 2 kb of right and 2.4 kb of left split-marker DNA fragments, respectively, and 0.6 kb of homologous sequence between these two fragments (Table S2).

Introduction of Cas9 RNP complexes into *P. ostreatus* protoplasts

To prepare Cas9 RNP targeting *pyrG*, 10 µg of Cas9 protein, 5 µg of the sgRNA (around 1:2 molar ratio), and 1× H buffer were mixed and assembled at 37 °C for 20 min. The assembled Cas9 RNP was mixed with 200 µl of the prepared protoplasts (8×10^6 protoplasts) in STC buffer. After that, the PTC buffer was added to the reaction, followed by the spread of the PEG/$CaCl_2$-mediated protoplasts onto a regeneration medium (RMUU, YMG with 0.5 M sucrose, uracil, and uridine) containing 0.1% (w/v) 5-FOA. After two weeks, the 5-FOA-resistant colonies, which may be *pyrG* mutants (Nakazawa et al., 2016b), appeared. The colonies were then transferred on to YMGUU plates containing 0.1% (w/v) 5-FOA.

To obtain hygromycin-resistant transformants (HDR-mediated *pyrG* disruptant), the treated protoplasts were mixed with the right and left donor DNA templates (2 µg each) and then spread onto the RMUU. After 24 h, 5 ml of RMUU containing hygromycin was overlaid on the same plate to obtain a final concentration of 150 µg/ml hygromycin. The hygromycin-resistant colonies that appeared were transferred to YMGUU plates with 100 µg/ml hygromycin, and then inoculated onto YMG plates containing 0.1% (w/v) 5-FOA. Further details regarding 5-FOA-resistant and hygromycin-resistant transformants are available in Nakazawa et al. (2016b) and Salame et al. (2012).

Genomic PCR experiments

For rapid genomic PCR experiments, fungal genomic DNA extraction and PCR techniques were conducted as described by Nakazawa et al. (2016b) and Boontawon et al. (2021). For PCR to identify *pyrG* mutations, the KOD FX Neo (Toyobo, Osaka, Japan) was used, followed by PCR fragment purification using the FastGene Gel/PCR Extraction Kit (Nippon Genetics) and Sanger sequencing.

RESULTS AND DISCUSSION

Cleavage of the target DNA sequence by Cas9 RNP *in vitro*

To conduct RNP-assisted gene mutation, Cas9 RNP was prepared by mixing the Cas9 protein and sgRNA targeting *pyrG* (Cas9/*pyrG*sg1). To examine the effectiveness of the assembled Cas9 RNP, *in vitro* digestion of the target DNA using Cas9 RNP was performed. First, the genomic fragment containing *pyrG* (1.1 kb containing the target sequence; at the nucleotide position −83 to +1032 from the start codon) was amplified using the primer pair MT10/MT11 (lane 1 in Fig. 1A; Table S1). If the Cas9 RNP cleaves the DNA fragment at the target site, this PCR-amplified fragment is digested to generate two fragments (approximately 0.5 kb and 0.6 kb fragments). As shown in Fig. 1B, the fragment was digested as expected, indicating that the Cas9/*pyrG*sg1 RNP complex efficiently cleaves double-stranded DNA at the target site *in vitro*.

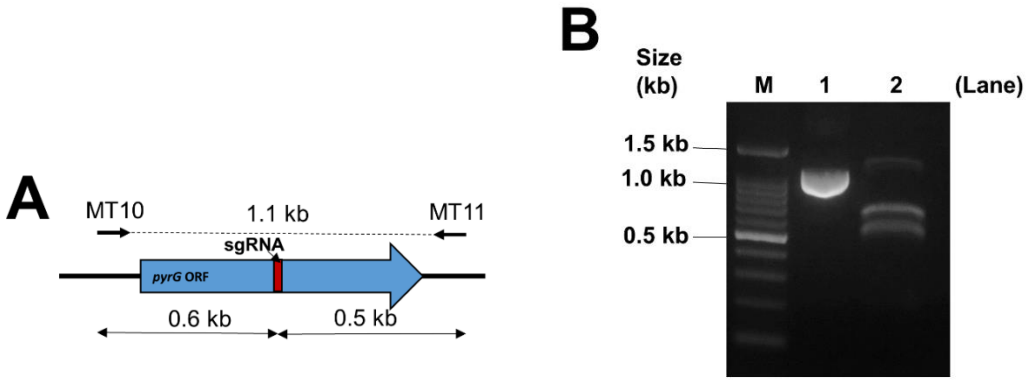

Fig. 1. *In vitro* cleavage assay using Cas9 RNP. (A) A schematic diagram showing *pyrG* locus, and (B) The visualization of digestion efficiency of Cas9/*pyrG*sg1 RNP complex on agarose gel electrophoresis.

Introduction of the Cas9 RNP can confer resistance to 5-FOA on the PC9 strain

pyrG was selected as the target to evaluate the efficiency of Cas9 RNP-assisted gene mutagenesis. Mutations in this gene confer resistance to 5-FOA on *P. ostreatus* (Nakazawa et al., 2016b), enabling a simple method to identify the mutant by examining resistance/sensitivity. The Cas9/*pyrG*sg1 RNP complex was introduced into the protoplasts of *P. ostreatus* PC9 (Table 1), followed by the spread of the protoplasts onto the RMUU containing 0.1% (w/v) 5-FOA. Seven 5-FOA-resistant strains were obtained by introducing Cas9/*pyrG*sg1 RNP (a 1:2 molar ratio of Cas9: sgRNA), but not by introducing Cas9 protein alone (Table 2). Similar results were obtained in the replicate experiments (Table 2), which showed that introducing Cas9 RNP generates 5-FOA-resistant (probably *pyrG* mutant) strains.

Table 2. Mutation frequencies of NHEJ repair in PC9 strain using Cas9 RNPs.

Cas9 protein (μg)	**sgRNA targeting *pyrG* (μg)**	**5-FOA-resistant strain**			***pyrG* mutant (NHEJ) by Sequencing**		
		Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
-	-	0	0	0	-	-	-
10	-	0	0	0	-	-	-
10	5	7	6	6	5/5	3/3	3/3

Identification of *pyrG* mutation in some of the 5-FOA-resistant strains

The author verified that the 5-FOA-resistant strains were *pyrG* mutants. Normally, CRISPR/Cas9 produces small insertions or deletions (indels) which are mediated by the NHEJ pathway. Deletion of a single nucleotide to several hundreds of nucleotides and insertion of several nucleotides can occur at or around a target site. Thus, the author first amplified a genomic fragment of the partial open reading frame (ORF) of *pyrG* (0.35 kb at the nucleotide position +402 to +757 from the start codon) containing the target sequence of sgRNA using the primer pair TB53/TB54. A 0.4 kb genomic fragment, which was amplified from the ORF of *mer3*, was amplified as a positive control using the primer pair FY15/FY16 (Yamasaki et al., 2021).

Five 5-FOA-resistant strains were selected to examine the mutations using PCR (Table 2). The PCR product was amplified from all five 5-FOA-resistant strains (lanes 1–5 in Fig. S1), and PC9 when the primer pair FY15/FY16 was used. As for the genomic fragment amplified using TB53/TB54, similar to the host strain PC9, the fragment was amplified from three of the five 5-FOA-resistant strains (lanes 3–5 in Fig. S1; strain py#3–5). Furthermore, the size of the fragments amplified from py#1–2 appeared to be different from those amplified from PC9 (lanes 1–2 in Fig. S1; strains py#1–2, respectively), suggesting that apparent indels may have

been introduced at the target site of *pyrG*, at least in py#1–2.

To verify whether small indels were introduced at the target site in the five 5-FOA-resistant strains (Fig. 2A; Table 1), the nucleotide sequences of the PCR products were determined using the primer TB53. The results revealed a 39-bp deletion and 37-bp insertion at the target site of *pyrG*sg1 in py#1 and #2, respectively. Then, 5-bp, 1-bp, and 1-bp deletions at the target site in py#3–5, respectively, were also identified (Fig. 2B; Table 1). This result indicates that there are *pyrG* mutants harbouring small indel mutations. Following *pyrG-1–7* designated in our previous studies (Nakazawa et al., 2016b; Boontawon et al., 2021), the newly identified mutations in py#1–5 were designated as *pyrG-8–11*, respectively (Table 1). This is the first example of introducing mutations in edible mushrooms using Cas9 RNP, which could be applied to the molecular breeding of cultivated strains that can be accepted more widely.

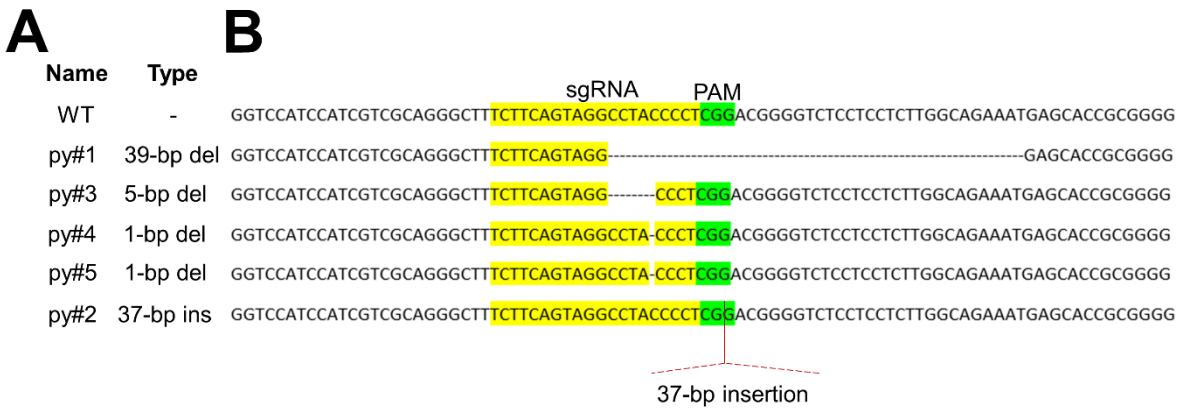

Fig. 2. Identification of *pyrG* mutation in (A) the five 5-FOA-resistant strains obtained after introducing Cas9 RNP. (B) Nucleotide sequences of *pyrG* locus were shown. For highlights in the nucleotide sequence: yellow shades indicate sgRNA, green shades indicate a protospacer adjacent motif (PAM) sequence, and dash lines indicate deletion.

Co-transformation of Cas9 RNP with the split-marker approach could efficiently disrupt targeted gene in non-*ku80* strains

In previous experiments, *pyrG* mutations were introduced by Cas9 RNP, which was mediated by NHEJ. However, the outcomes of NHEJ-mediated mutations are unpredictable. For precise gene deletion/replacement, homology-directed repair (HDR) is generally used in basic fungal research. A split-marker technique has been demonstrated in *Saccharomyces cerevisiae* (Fairhead et al., 1996; 1998). This method uses two truncated but overlapping selection markers on each side of the constructs and flanked by a homologous DNA sequence; only the transformant with an intact or functional selection marker gene, which is generated via HDR, can grow on a selective medium. The split-marker approach has been suggested to

increase the frequency of targeted gene disruption via homologous recombination by as much as 100% (Choquer et al., 2005; You et al., 2009; Lin and Chung, 2010). Currently, this method has been introduced in some ascomycetous filamentous fungi such as *Cochliobolus heterostrophus, Gibberella zeae*, *Cercospora nicotianae*, and *Fusarium oxysporum* (Catlett et al., 2003; You et al., 2009; Liang et al., 2014), but not in agaricomycetous fungi.

To examine whether the split-marker approach can be used in the wild-type strain of *P. ostreatus*, the author introduced split-marker DNA fragments into protoplasts of the *P. ostreatus* PC9 strain. First, split-marker DNA templates alone (without Cas9 RNP) were introduced as a control for *P. ostreatus* PC9. Only one hygromycin-resistant transformant was obtained. This strain grew on YMGUU with 0.1% (w/v) 5-FOA (Table 3), suggesting that it is a *pyrG* disruptant and that the split-marker DNA template alone could be used to generate 5-FOA-resistant transformants for the *P. ostreatus* PC9 strain. A replicate experiment was performed (Table 3). However, only a small number of transformants were obtained with this approach.

Recently, Boontawon (2021) reported that gene replacement in *fcy1* via HDR using a plasmid-based CRISPR/Cas9 in the *ku80*-deficiency *P. ostreatus* strain 20b was efficiently introduced. Furthermore, Vonk et al. (2019) demonstrated an efficient Cas9 RNP-assisted gene replacement using a conventional donor DNA template in *S. commune*. The author hypothesised that efficient gene replacement can be performed in wild-type *P. ostreatus* using the split-marker approach together with Cas9 RNP. In this study, to examine the efficiency of gene replacement using these techniques, the Cas9 RNP along with the split-marker DNA template was introduced into protoplasts from PC9. In addition, to optimise the Cas9 RNP system, different concentrations of Cas9/*pyrG*sg1 RNP (5 μg, 10 μg, and 20 μg of Cas9 protein) were used for transformation. Two out of two (100%), nine out of 18 (50%), and four out of 15 (27%) hygromycin-resistant transformants were obtained by introducing 5 μg, 10 μg, and 20 μg of Cas9/*pyrG*sg1 RNP, respectively, along with the donor DNA template, exhibited resistance to 5-FOA (Table 3). Thus, highest frequency of 5-FOA-resistant strains, which may be *pyrG* mutants, was obtained when 10 μg of the Cas9/*pyrG*sg1 RNP was introduced into the protoplasts of *P. ostreatus* PC9 strains along with the split-marker repair templates. To ensure reproducibility, a replicate experiment was performed under the best conditions (Table 3). Wang et al. (2018) reported that the highest efficiency of gene disruption in *Fusarium oxysporum* was obtained when 10 μg of Cas9 RNP was used; however, a lower (5 μg) or higher (15 and 20 μg) concentration of the Cas9 RNP did not yield a higher efficiency of gene disruption. The author hypothesised that different amounts of Cas9 RNP may affect the

efficiency of gene disruption, as Kouranova et al. (2016) previously showed that a high ratio of Cas9 RNP to the targeted DNA resulted in low activity of protein specificity and/or inefficient complex formation between them.

Next, the author verified that the *pyrG* sequence was replaced with the introduced donor DNA. The 16 5-FOA-resistant strains obtained by introducing split-marker DNA templates with or without the Cas9/*pyrG*sg1 RNP complex were selected for examination of gene mutations using genomic PCR (Table 3). First, the author determined whether genomic fragments containing 1.5 kb of the 5′ arm and 1.4 kb of the 3′ arm were amplified from these 5-FOA-resistant strains with the primer pairs TB74/TB75 and TB76/TB77, respectively (Fig. 3): TB74 and TB77 were designed to hybridize to the 5′-upstream and 3′-downstream of the *pyrG* disrupting cassette, respectively. TB75 and TB76 were designed in the hygromycin resistance gene in the disruption cassette. These PCR fragments were anticipated to be amplified from 5-FOA-resistant strains in which the HDR-mediated *pyrG* mutation was introduced, but not from the PC9 wild-type strain. As shown in Fig. S2A, the expected fragments were amplified from the 13 5-FOA-resistant strains when both primer pairs TB74/TB75 and TB76/TB77 were used (lanes 1–3, 5–10, 12, and 14–16; strains sr#1–3, 5–10, 12, and 14–16; Tables 1 and 3), but not from the wild-type strain (lane WT). Next, the other primer pair TB53/TB54 was used to verify whether a 0.4 kb PCR product was amplified from the wild-type strain, but not from the 5-FOA-resistant strains (Fig. 3). As shown in Fig. S2B, the expected fragment was amplified from the PC9 strain (lane WT), but not from the 13 5-FOA-resistant strains (lanes 1–3, 5–10, 12, and 14–16; strains sr#1–3, 5–10, 12, and 14–16). These results suggest that HDR-mediated gene replacement was efficiently introduced into the genomes of the 13 5-FOA-resistant strains when the Cas9/sgRNA RNP together with the split-marker approach was used. In *P. ostreatus*, when the gene targeting technique was used with the wild-type strain PC9, only 2.3% correct homologous integration was obtained (Salame et al., 2012), which may be due to the relatively high NHEJ activity in *Agaricomycetes*. In contrast, with this Cas9 RNP approach, the gene in 7%–100% of the samples was successfully disrupted in the wild-type strain PC9, which would likely be applicable to many other agaricomycetes without generation of NHEJ-deficient strains by disruption of *ku70*, *ku80*, or *lig4*.

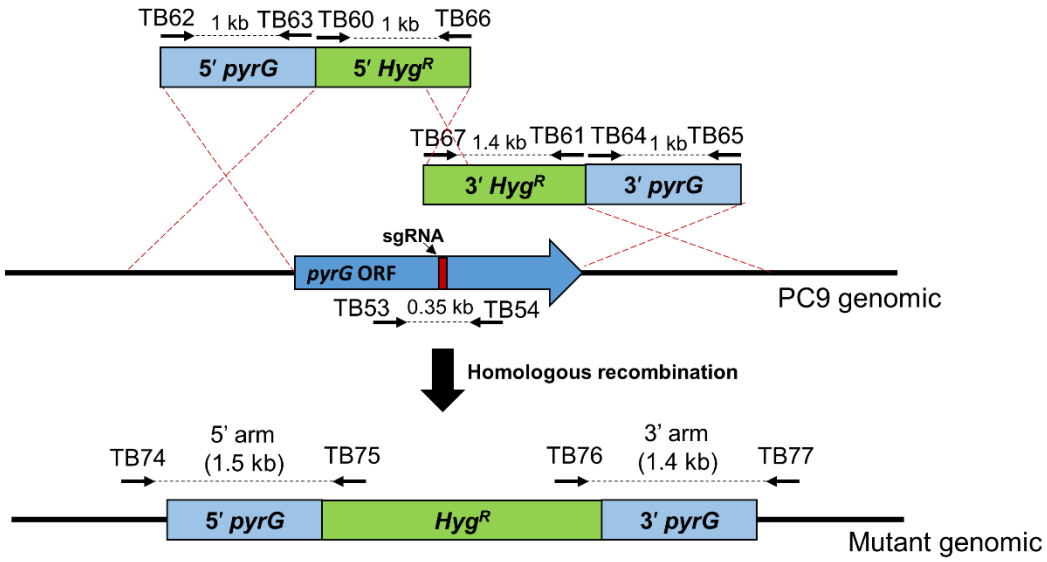

Fig. 3. Targeted gene deletion using the split-marker recombination along with the Cas9 RNP. (A) A schematic diagram of the protocol used to generate the repair templates. (B) A schematic diagram showing the *pyrG* disruption using Cas9 RNP and the donor DNA. Black arrows show the primer pairs used for the genomic PCR in Fig. S1–2.

Table 3. Gene disruption frequencies of homologous directed repair in PC9 strain using Cas9 RNP.

Strain	Cas9 protein (μg)	sgRNA targeting *pyrG* (μg)	Split-marker donor DNA (μg)	hygromycin-resistant strain		5-FOA-resistant strain		*pyrG* disruptant (HDR) by PCR	
				Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
PC9	-	-	2	1	2	1 (100%)[1]	1 (50%)[1]	1 (100%)[2]	1 (50%)[2]
PC9	5	2.5	2	2	-	2 (100%)[1]	-	2 (100%)[2]	-
PC9	10	5	2	18	10	9 (50%)[1]	4 (40%)[1]	8 (44%)[2]	3 (30%)[2]
PC9	20	10	2	15	-	4 (27%)[1]	-	2 (13%)[2]	-

[1]Percentage of 5-FOA-resistant strains from the total number of obtained hygromycin-resistant strains; [2]Percentage of *pyrG* mutants from the total number of obtained hygromycin-resistant strains.

SUMMARY

In conclusion, this is the first report demonstrating genome editing using the Cas9 RNP system in the cultivated mushroom *P. ostreatus*. Cas9 RNP-assisted gene mutagenesis is capable of efficient and specific mutagenesis for molecular breeding purposes. This is the first step for molecular breeding of the cultivated strains that may be not classified as GMOs, and thus, can be more widely accepted by the consumers. Furthermore, Cas9 RNP-assisted gene replacement using split-marker recombination was also demonstrated in the *P. ostreatus* wild-type strain. This approach can resolve several problems in NHEJ-deficient strains, as well as difficulty in carrying out molecular genetic studies using various non-model agaricomycetes.

SUPPLEMENTARY DATA

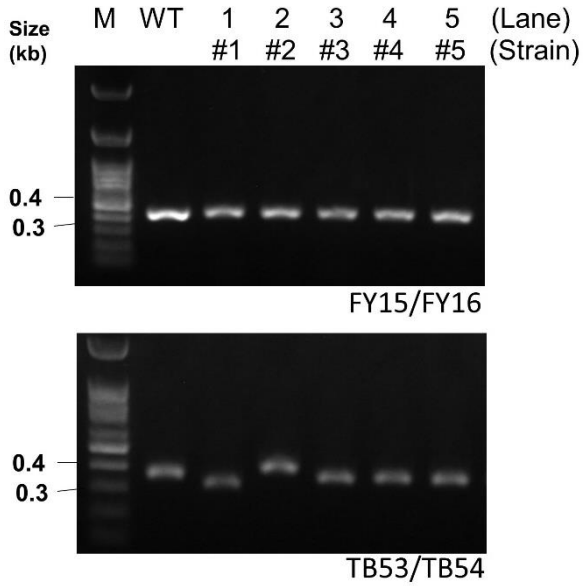

Fig. S1. Identification of *pyrG* mutation in the five 5-FOA-resistant strains obtained by introducing Cas9-*pyrG*sg1 RNP complex in *P. ostreatus* PC9 strain. Genomic PCR experiments identify *pyrG* mutation. Lane WT, parental strain PC9 used as a control; lanes 1–5, 5-FOA-resistant strains; lane M, a 100 bp molecular weight marker (0.1–1.5 kb) or a 1 kb DNA ladder plus (0.1–10.0 kb). For more details regarding the estimated lengths of the PCR products amplified from the genome, see Table S2.

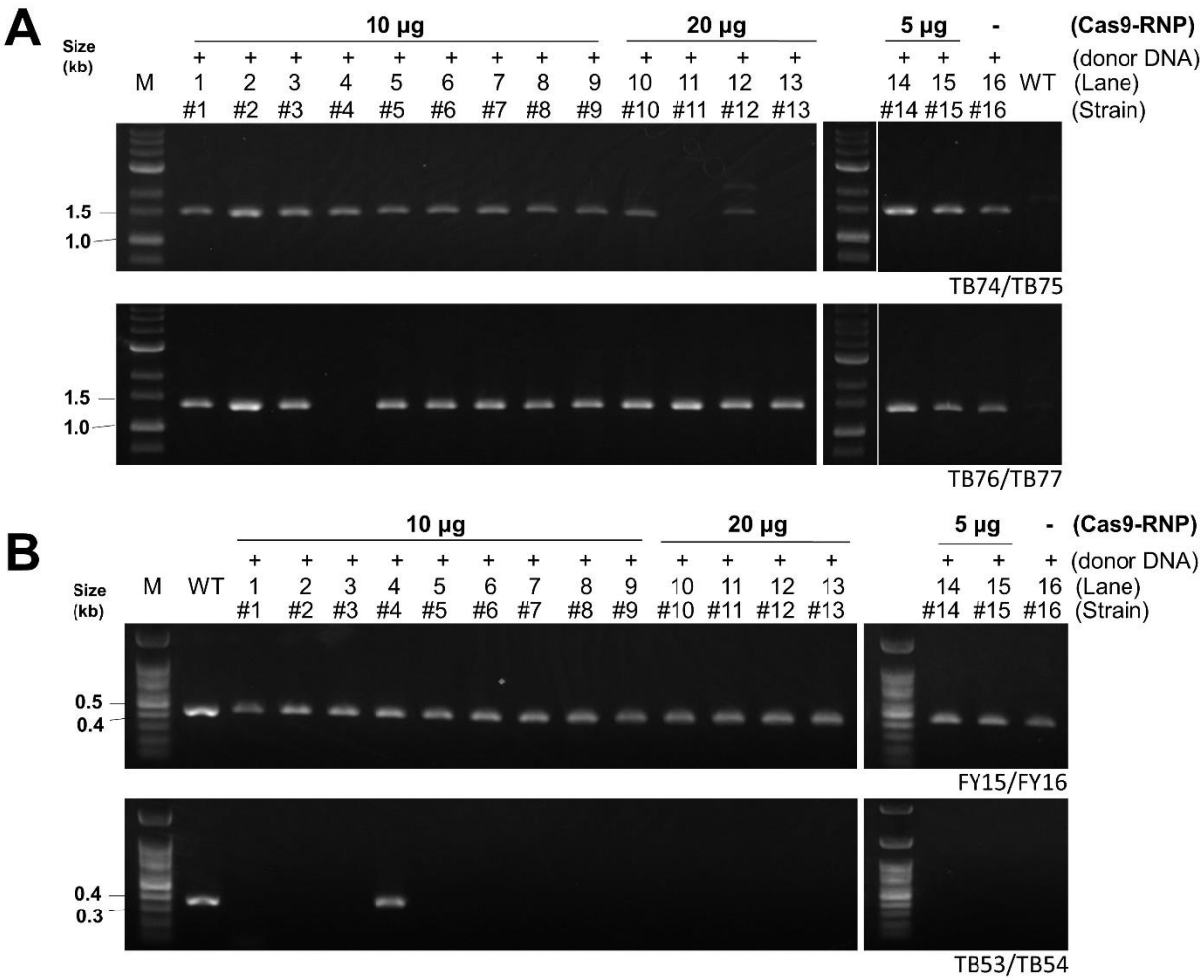

Fig. S2. Identification of *pyrG* mutation in the 16 5-FOA-resistant strains obtained by introducing Cas9-*pyrG*sg1 RNP and/or the split-marker donor DNA in *P. ostreatus* PC9 strain. Genomic PCR experiments identify *pyrG* deletion. Lane WT, parental strain PC9 used as a control; lanes 1–16, 5-FOA-resistant strains; lane M, a 100 bp molecular weight marker (0.1–1.5 kb) or a 1 kb DNA ladder plus (0.1–10.0 kb). For more details regarding the estimated lengths of the PCR products amplified from the genome, see Table S2.

Table S1. Primer pairs used in this study.

Primer	Sequence (5' to 3')
CI78	AAAAAAAAAGCACCGACTCG
FY15	AACCCCAAGTTCTATGCGTTG
FY16	ACTTACTCTTCTGCAGCCGAC
MT10	TTCGCAGCCCCTTTCTTTCACTAAG
MT11	ACGCGGAAACCAATCTTTGTGC
TB41	GATTGTCTTCAGTAGGCCTACCCCT
TB42	AAACAGGGGTAGGCCTACTGAAGAC
TB53	CGCCGCTTGTAGGAAACACAG
TB54	CTGCTGCCCCATACCATCTCC
TB60	CGCCAGGGTTTTCCCAGTCACGACTTCATTTAAACGGCTTCACGGGC
TB61	AGCGGATAACAATTTCACACAGGACAATATTCATCTCTCTCCATCG
TB62	CCACTTCACCAAGCTGTG
TB63	GTCGTGACTGGGAAAACCCTGGCGATGGTTGAGAGTAGGTGCCTTC
TB64	TCCTGTGTGAAATTGTTATCCGCTAATTGGTTCATTAAACTAGACAAGC
TB65	CTAGCAAACGCCACGAGGAGG
TB66	CATTGTTGGAGCCGAAATCC
TB67	GTCTGTCGAGAAGTTTCTGATC
TB68	CTAATACGACTCACTATAGTCTTCAGTAGGCCTACCCCTGTTTCAGAGCTATGCTGGAAA
TB69	AAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTA GCCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGAAAC
TB70	AAAAAGCACCGACTCGGTGCC
TB71	CTAATACGACTCACTATAGTCTTCAGTAGGCCTACCC
TB74	CATCAGTCGCAGGACCAGAC
TB75	GGAGATGGATTGGACACGAAG
TB76	CCACCTACCTCCACCACCTAC
TB77	CGTATAGTTACTGAGCCGATGC

Table S2. Estimated lengths of the PCR fragments that were amplified from each strain.

Primer set	**Strain/ Function**	**Estimated length (kb)**
TB41/TB42	*pyrGs*g1	-
FY15/FY16	wild-type / positive control	0.4
	mutant / positive control	0.4
TB53/TB54	wild-type	0.35
	pyrG mutant	Small indels of nucleotide, or not amplified
TB60/TB61	*hph* cassette	1.9
TB62/TB63	5'-upstream of *pyrG*	1.0
TB64/TB65	3'-downstream of *pyrG*	1.0
TB62/TB65	intact donor DNA template	3.9
TB62/TB66	right arm of split-marker template	2.0
TB65/TB67	left arm of split-marker template	2.4
TB74/TB75	5' arm for checking *pyrG* disruption	1.5
TB76/TB77	3' arm for checking *pyrG* disruption	1.4
TB68/TB69/TB70	Construction of sgRNA scaffold for *pyrG*	0.3
TB71/CI78	Amplification of *pyrG* sgRNA scaffold	0.3
MT10/MT11	Amplification of ORF of *pyrG*	1.1

CONCLUSIONS

Until recently, classical breeding has been used to generate improved commercial mushroom cultivars; however, classical breeding remains to be laborious and time-consuming (Sonnenberg et al. 2008). In this thesis, efficient genome editing using the introduction of CRISPR/Cas9 plasmid, and the Cas9 RNP for assisting targeted gene mutagenesis and gene disruption via NHEJ and HDR mechanisms were successfully established. These techniques may be also applied for molecular breeding to open the door for non-GMO genome editing in the cultivated edible mushrooms.

Now, molecular breeding, which is more efficient and straightforward than the conventional breeding, can be conducted for improving strains with desired traits. Using the plasmid expressing Cas9 and sgRNA, the author has successfully developed an efficient gene targeting method in *P. ostreatus*. The 5-FC- and 5-FOA-resistant *P. ostreatus* strains were obtained when introduced pCcPef3-126 plasmid (Sugano et al., 2017) targeting *fcy1* and *pyrG*, respectively. The result revealed that the obtained strains were either *fcy1* or *pyrG* mutant harboring small indel mutations at the target sites. The large insertional mutations from an introduced plasmid were also identified in *fcy1*/*pyrG* mutant strains. Furthermore, a precise gene replacement via homologous recombination using CRISPR/Cas9 and donor DNA templates (homology arms of 0.2, 0.5, or 1 kb) were introduced into *P. ostreatus* strain 20b. The efficiency of gene replacement was higher when using longer length of homology arms. This study demonstrated that the most efficient CRISPR/Cas9 (20–94.9%) was established in *P. ostreatus*, when compared with the other agaricomycetes (10.5–25.8%) including *C. cinerea* and *G. lucidum* (Sugano et al., 2017; Wang et al., 2020).

Understanding the molecular mechanisms controlling dikaryon formation in *Agaricomycetes,* which is basically controlled by *A* and *B* mating-type loci, contributes to improving mushroom cultivation and breeding (Raudaskoski, 2015). However, mechanisms on fruiting body formation have been intensively investigated only in model mushrooms such as *C. cinerea* and *S. commune* (Specht, 1995; Inada et al., 2001). To extend the knowledge to the other non-model mushrooms, the author clarified the functions of *pcc1* and *clp1* on sexual development in *P. ostreatus* using CRISPR/Cas9 tool (Chapter 1). The results suggest that the transcriptional expression of *Popcc1* and *Poclp1* are under the control of the *A*- and/or *B*-regulated pathways. In *Popcc1* mutant strains, pseudoclamp cells were identified, suggesting that *A*-regulated clamp cell formation was not induced in monokaryons, but cause defects in *B*-regulated clamp connection. In *Poclp1* mutant strains, clamp connection was not observed

when crossing between the two compatible *Poclp1* mutant strains. Thus, *clp1* mutations cause defects in dikaryosis in *P. ostreatus*.

In cultivated mushrooms, a gene targeting technique to generate non-GM strain was lacking, here, the Cas9 RNP was used to induce gene mutagenesis in *P. ostreatus*. The 5-FOA-resistant strains were obtained when introduced Cas9 RNP targeting *pyrG*. The result suggests that these drug-resistant strains were *pyrG* mutants with small indel mutations. This technique would be used to improve edible mushroom strains that more widely accepted by the customers. Furthermore, Cas9 RNP with the split-marker donor DNA template could efficiently replace targeted gene in non-*ku80* strain. This system could overcome many disadvantages of NHEJ-deficient strain for example difficulty in fruiting body formation and development in *C. cinerea ku70* and *lig4* disruptant strains, respectively (Nakazawa et al., 2011b).

In conclusion, the present thesis demonstrated efficient genome editing using the plasmid-based CRISPR/Cas9 and the Cas9 RNP system in the worldwide popular cultivated mushroom for the first time. Furthermore, essential functions of *pcc1* and *clp1* in fruiting body development in *P. ostreatus* were demonstrated. In the future, it is desirable to generate non-GM edible mushroom strains using the Cas9 RNP technique.

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LIST OF PUBLICATIONS

ORIGINAL PAPERS

Boontawon T, Nakazawa T, Inoue C, Osakabe K, Kawauchi M, Sakamoto M, Honda Y (2021) Efficient genome editing with CRISPR/Cas9 in *Pleurotus ostreatus*. AMB Expr. 11(1):30. doi:10.1186/s13568-021-01193-w.

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[1]Laboratory of Forest Biochemistry, Division of Environmental Science and Technology, Graduate School of Agriculture, Kyoto University
[2]Laboratory of Terrestrial Microbial Ecology, Division of Environmental Science and Technology, Graduate School of Agriculture, Kyoto University
[3]Laboratory of Environmental Interface Technology of Filamentous Fungi, Division of Environmental Science and Technology, Graduate School of Agriculture, Kyoto University
[4]Department of Biotechnology, Faculty of Science, Mahidol University, Thailand
[5]Kyoto International Student Orientation Center (Satsuki dormitory), Kyoto Prefectural Government