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ORIGINAL ARTICLE

Marker-free genome editing in the edible mushroom, *Pleurotus ostreatus,* using transient expression of genes required for CRISPR/Cas9 and for selection

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Abstract

In a previous study, we reported a transient transformation system using repeated screening for hygromycin B (Hyg) resistance in the basidiomycete *Ceriporiopsis subvermispora*. In the present study, by combining this technique with CRISPR/Cas9, we demonstrated successful marker-free genome editing in *Pleurotus ostreatus*, which is one of the most economically important cultivated mushrooms as well as a model white-rot fungus. At first, transformant selection mediated by the transient expression of marker genes was demonstrated using a plasmid harboring the Hyg resistance gene (*hph*) in *P. ostreatus*. Then, genome editing of *fcy1*, which confers 5-fluorocytosine (5-FC) resistance to the host cell, was performed by the transient expression of Cas9, gRNA, and *hph* and strains with 5-FC resistance and Hyg sensitivity were isolated. Additionally, genome editing of *fcy1* in these strains was confirmed by Sanger sequencing. To our knowledge, this is the first report of marker-free genome editing through the transient expression of Cas9, gRNA, and *hph* in agaricomycetes, which opens the door for repeated genome editing in these fungi.

Keywords: Transient transformation, Genome editing, Agaricomycete, Mushroom, Wood-rot fungi, *Pleurotus ostreatus*, CRISPR/Cas9

Introduction

Genetic transformation is a basic tool for the functional analyses of the genes of interest, as well as for molecular breeding. Generally, genetic transformations can be divided into stable and transient transformations [1, 2]. In stable transformation, the foreign DNA is mostly integrated into the host chromosome. In the case of transient transformation, the introduced DNA resides temporarily in the cell and is subsequently lost because chromosome integration does not occur. Both transformation methods have been used for genetic investigations in plants and animal cells. In filamentous fungi, stable transformation has been widely used to integrate genes at random or targeted sites on chromosomes. A substantial number of different selection markers are required for multiple gene integrations or disruptions, limiting such genetic manipulation. Transformation of *Schizophyllum commune* and *Coprinopsis cinerea* was first reported in basidiomycetes [3, 4]. Stable transformation has been developed for the genetic investigations of several edible mushrooms [5–7]. Recently, we reported the first transient transformation of the basidiomycete *Ceriporiopsis* (*Gelatoporia*) *subvermispora* [8]. Transient transformants carrying temporary hygromycin B (Hyg) resistance were obtained only when a functional promoter was used to express the coding

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sequence of the Hyg resistance gene (*hph*). This result demonstrated that the unstable Hyg-resistant colonies were not background colonies but were dependent on the transient expression of the introduced recombinant *hph*. If this system can be applied to other fungi, repetitive use of the selection marker for multiple transformations becomes possible, which opens the door for the multiple gene disruptions or insertions.

Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) has become the most popular genome-editing technology [9]. CRISPR/Cas9 was originally found in bacteria and archaea which functions as an adaptive immune system against invading nucleic acids. In this system, a 20-nt guide RNA (gRNA) recruits the Cas9 protein to the target site, and Cas9 cuts the polynucleotide linkages to make a double-strand break in the host genome. During the break repair process, a mutation, such as the deletion or insertion of a nucleotide sequence, may occur in the host genome. In the presence of donor DNA containing flanking sequence surrounding the double-strand break, a gene knock-in can occur through homologous recombination at the gRNA target site. CRISPR/Cas9 was successfully adapted for the agaricomycete *C. cinerea* in 2017 [10]. Recently, we developed the first CRISPR/Cas9 systems for edible mushrooms using *P. ostreatus* [11, 12].

White-rot fungi, including *P. ostreatus,* have a unique ability to depolymerize wood lignin. Generally, it is difficult to effectively remove lignin from woody biomass under mild conditions to obtain polysaccharides for biorefineries. Therefore, the molecular mechanism underlying lignin biodegradation by white-rot fungi is of interest to establish new methods for efficient and ecofriendly lignin removal from wood biomass [13]. Multiple genes encoding 'ligninolytic enzymes' have been predicted in the *P. ostreatus* genome [14]. In the case of extracellular peroxidase, three genes encoding versatile peroxidases (VPs) and six genes encoding manganese peroxidases (MnPs) were predicted in the *P. ostreatus* genome [15]. To date, there have been no reports directly demonstrating that any of these extracellular peroxidases are involved in lignin degradation by white-rot fungi. Salame et al. isolated single gene disruptants of *mnp* or *vp* using gene knock-out in a non-homologous end joining (NHEJ)-deficient strain, but these strains did not show a drastic decrease in their lignin degradation ability on wood sawdust medium [16–18]. These results suggest that the enzymes encoded by the disrupted genes are not essential for ligninolysis, but it is important to consider the redundancy of these genes and their effect on the phenotype of the single *mnp* or *vp* disruptants. Therefore,

multiple gene disruptions are required to understand the physiological function of each ligninolytic enzyme.

To knock-out a gene on a chromosome, conventional gene targeting through homologous recombination-based replacement of a target site with a selectable marker gene is required. As available selection markers are limited, multiple gene targeting is very difficult in this fungus [19]. This limitation has hindered efforts to disrupt multiple genes encoding lignin-degrading enzymes. Recently, CRISPR/Cas9-directed genome editing was used to introduce mutations at targeted sites on the chromosome and to isolate disruptants of a gene of interest. However, in this case, a selection marker is still essential to isolate the mutant strains, and there is a limitation for repetitive genome-editing experiments owing to the shortage of available selection markers. If a practical method for temporal CRISPR/Cas9 and marker selection could be established, repetitive genome editing would become available, permitting multiple gene disruptions of ligninolytic enzyme genes to elucidate their physiological functions in wood degradation by *P. ostreatus*.

In this study, marker selection of the transient transformants dependent on the temporal expression of *hph* was established in *P. ostreatus*. Then, marker-free genome editing was performed by combining the transient expression of Cas9, gRNA, and *hph*.

Materials and methods
Strains, plasmids, culture conditions, and genetic techniques
Pleurotus ostreatus monokaryon strain PC9 (Spanish Type Culture Collection accession number CECT20311) was used for transformation in this study [20]. The 5-fluorocytocine (5-FC) sensitivity of this strain was confirmed in our previous study [21]. The strains used in this study are listed in Table 1. Yeast and malt extract with glucose (YMG) agar were used for routine culture [22]. The cultures were maintained at 28 °C in continuous darkness unless otherwise stated.

Transformations using the hygromycin B resistance gene (*hph*) were performed as described previously [16]. For the selection of transformants with *hph*, YMG supplemented with 100 µg/mL hygromycin B (YMG-Hyg) was used. YMG supplemented with 0.1% (w/v) 5-FC (YMG-5FC) was used to select for the *fcy1* mutant.

The plasmids pPHT1 [23], pCcPef3-126 [24], and pCcPef3-126-fcy1sg2 [11] were constructed as previously described.

Rapid colony PCR and sequencing
Crude genomic DNA was prepared as described previously [25]. The PCRs described below were performed

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Table 1 *Pleurotus ostreatus* strains used in this study

Strain	Genotype/description	Source
PC9	*A2B1*	[20]
PHT1-1	*A2B1* Hyg[Ra] strain obtained after introducing pPHT1	This study
PHT1-2	*A2B1* Hyg[R] strain obtained after introducing pPHT1	This study
PHT1-3	*A2B1* Hyg[Sb] strain obtained after introducing pPHT1	This study
PHT1-4	*A2B1* Hyg[S] strain obtained after introducing pPHT1	This study
PHT1-5	*A2B1* Hyg[S] strain obtained after introducing pPHT1	This study
Pef3-1	*A2B1* 5-FC[Rc] Hyg[R] strain obtained after introducing pCcPef3-126-fcy1sg2	This study
Pef3-2	*A2B1* 5-FC[R] Hyg[R] strain obtained after introducing pCcPef3-126-fcy1sg2	This study
Pef3-3	*A2B1* 5-FC[R] Hyg[S] strain obtained after introducing pCcPef3-126-fcy1sg2	This study
Pef3-4	*A2B1* 5-FC[R] Hyg[S] strain obtained after introducing pCcPef3-126-fcy1sg2	This study
Pef3-5	*A2B1* 5-FC[R] Hyg[S] strain obtained after introducing pCcPef3-126-fcy1sg2	This study
Pef3-6	*A2B1* 5-FC[R] Hyg[S] strain obtained after introducing pCcPef3-126-fcy1sg2	This study

[a] Hyg[R] indicates hygromycin B-resistant strains

[b] Hyg[S] indicates a hygromycin B-sensitive strain

[c] 5-FC[R] indicates 5-fluorocytocine-resistant strain

Table 2 Primers used in this study

Primer	Sequence (5′ > 3′)
Cas9check_F2	GACGATGTTGACCTGAGGCA
Cas9check_R2	TTCGACCAGTCCAAGAACGG
hph_F	CTATTCCTTGCCCTCGGACGAGTGC
hph_R	ACTCACCGCGACGTCTGTCGAGAAG
TB9	CATTGCTTTGGCGGTTCAAACG
TB10	GCGTACCTCAGCGTCGTGTAC

using PrimeSTAR GXL DNA polymerase (Takara Bio) or KOD FX neo DNA polymerase (TOYOBO, Osaka, Japan). The primers used for the colony PCR are listed in Table 2.

The *cas9* insertion was confirmed by amplifying the 2232-bp fragment of the *cas9* gene using PCR with primers Cas9check_F2 and Cas9check_R2. The *hph* insertion was confirmed by amplifying the 1012-bp fragment of the *hph* gene using PCR with primers hph_F and hph_R. Genome editing of *fcy1* was detected by PCR with primers TB9 and TB10, and Sanger sequencing was performed against the resulting PCR product using the same primer sets (Fig. 3).
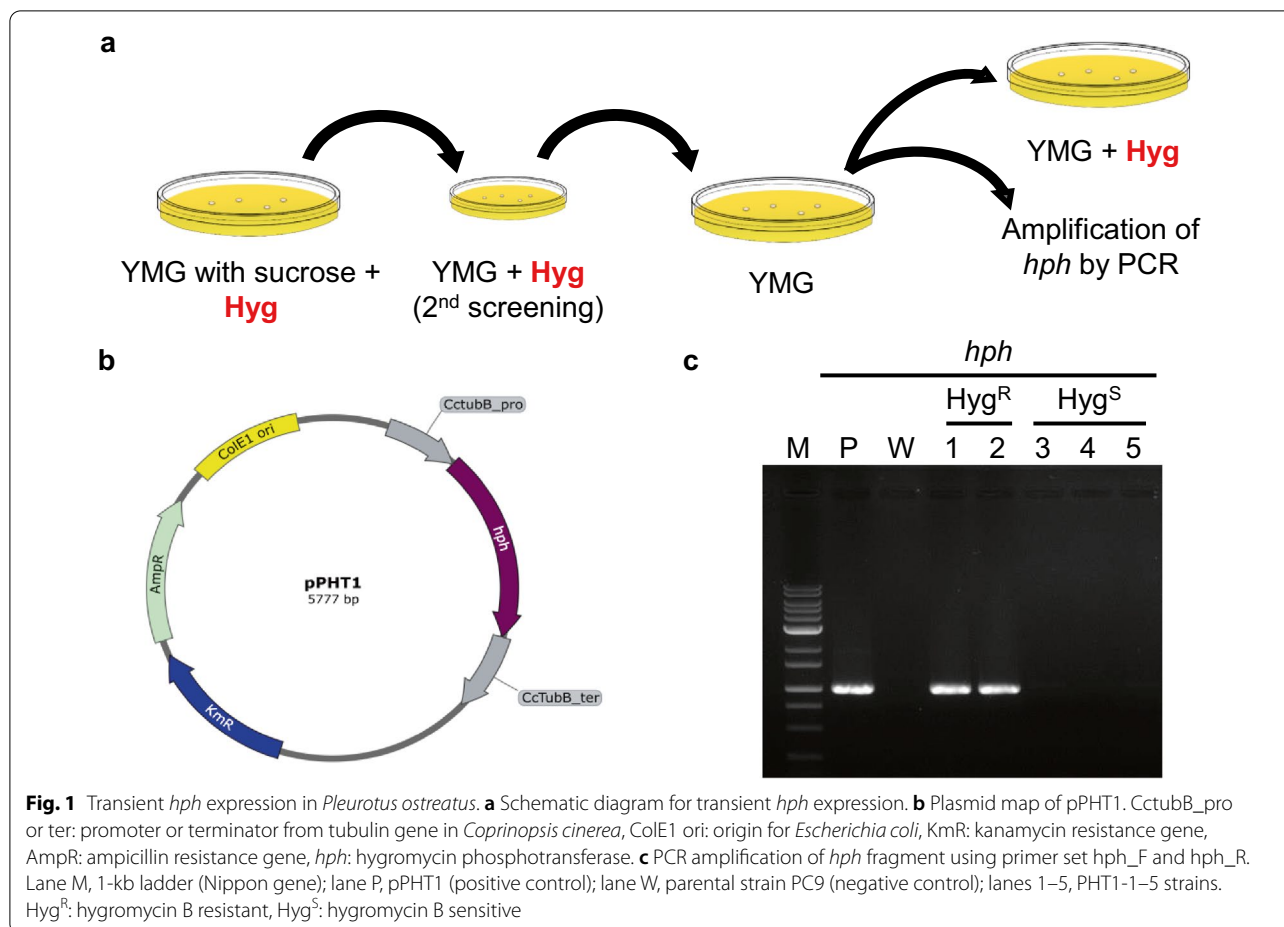
Results and discussion

Selection of transformants through transient expression of *hph* in *P. ostreatus*

Previously, transient expression of a selection marker was observed by introducing a plasmid harboring an *hph* expression cassette into *C. subvermispora*, which can be used for initial selection of transformants [8]. If transient *hph* expression can be used for the initial selection of transformants in *P. ostreatus*, marker-free genome editing will be possible in combination with the transient expression of Cas9 and gRNA. To determine whether transient *hph* expression could be used for initial selection of transformants in *P. ostreatus*, the pPHT1 plasmid harboring an *hph* expression cassette was introduced into wild-type *P. ostreatus*. A scheme of the transient expression of *hph* by introduction of pPHT1 is shown in Fig. 1a, b. In the first screening, transformants were selected on YMG-Hyg agar medium. From this plate, 46 viable strains were isolated and transferred to fresh YMG agar medium. After growth to reach the edge of the agar plate, transformants were passaged on YMG-Hyg agar medium to test for Hyg resistance. Three of the 46 strains showed loss of Hyg resistance. The presence of the *hph* gene in these strains was tested using colony PCR. The results showed that *hph* was amplified in Hyg-resistant strains, whereas no bands were detected in these three strains (Fig. 1c). These results indicate that temporal expression of *hph* occurred in *P. ostreatus* and could be applied to the initial selection of transformants. It is notable that transient *hph* expression can be used for initial screening of transformants in two taxonomically separated species, *P. ostreatus* and *C. subvermispora*, suggesting the possibility of a similar selection system in other basidiomycete fungi.

Marker-free genome editing by transient expression of Cas9, gRNA, and *hph*

In the previous study, we have developed CRISPR/Cas9 system for *P. ostreatus* [26]. However, it has not indicated that if genome editing is available via the transient expression of Cas9 and gRNA. Additionally,

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Fig. 1 Transient *hph* expression in *Pleurotus ostreatus*. **a** Schematic diagram for transient *hph* expression. **b** Plasmid map of pPHT1. CctubB_pro or ter: promoter or terminator from tubulin gene in *Coprinopsis cinerea*, ColE1 ori: origin for *Escherichia coli*, KmR: kanamycin resistance gene, AmpR: ampicillin resistance gene, *hph*: hygromycin phosphotransferase. **c** PCR amplification of *hph* fragment using primer set hph_F and hph_R. Lane M, 1-kb ladder (Nippon gene); lane P, pPHT1 (positive control); lane W, parental strain PC9 (negative control); lanes 1–5, PHT1-1–5 strains. Hyg^R: hygromycin B resistant, Hyg^S: hygromycin B sensitive

it is unsure whether transient expression of selection marker can be used for initial selection of genome-edited strains. To examine gene knock-out by transient Cas9, gRNA, and *hph* expression, the *fcy1* gene was selected as a target because loss of its function confers resistance to 5-FC in *P. ostreatus* [21]. This allowed us to identify mutants easily and efficiently by examining resistance/sensitivity to 5-FC after screening for temporal Hyg resistance during the initial screening. A schematic of the experimental design is shown in Fig. 2a. The plasmid pCcPef3-126-fcy1sg2, which was constructed for genome editing of *fcy1* in a previous study [11], was introduced into *P. ostreatus*, and transformants were selected on YMG-Hyg with sucrose agar medium (Fig. 2b). The obtained Hyg-resistant transformants were subcultured on YMG-5FC agar medium and subjected to the following analysis. From these transformants, 130 5-FC-resistant transformants were selected and passaged on fresh YMG agar media. After repeated passaging, strains were tested on YMG-Hyg and YMG-5FC agar media. Four of the 130 strains were 5-FC-resistant with a Hyg-sensitive phenotype

(Table 3). No *hph* amplicon was observed in one of the four Hyg-sensitive strains (Fig. 2c). Therefore, transient *hph* expression can be used to select the transformants under the Cas9 expression.

Insertion of *cas9* and genome editing of this Hyg-sensitive strain (Pef3-5) was confirmed. First, the *cas9* amplicon was not observed following the colony PCR of the Pef3-5 strain (Fig. 3a). Next, amplification of a targeted site of gRNA in the *fcy1* gene displayed a longer amplified fragment in Pef3-5 than in the wild-type strain (Fig. 3b, c). Sanger sequencing revealed an inserted sequence at the target site of the gRNA in the *fcy1* gene (Fig. 3d, e). The first 37-bp could not be identified for sequence origin. Most of this fragment perfectly matched a small portion of the *hph* expression cassette. These results suggest that genome editing of the *fcy1* gene in the Pef3-5 strain is mediated by the transient expression of Cas9, gRNA, and *hph*.

This is the first example of marker-free genome editing by transient Cas9, gRNA, and *hph* expression in basidiomycetes. As *cas9* is not integrated into the genome of the resulting strain (Fig. 3), our system should reduce
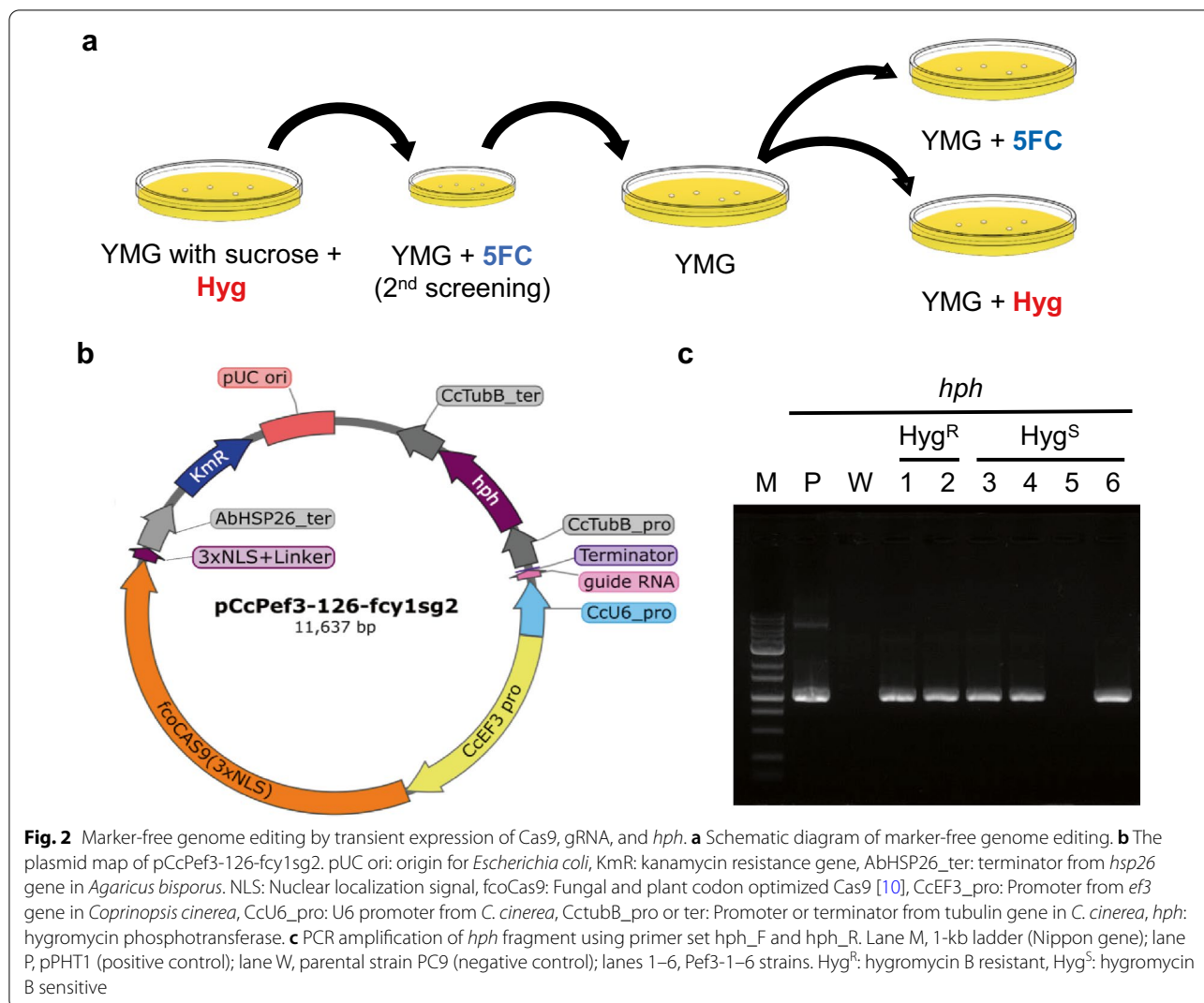
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Fig. 2 Marker-free genome editing by transient expression of Cas9, gRNA, and *hph*. **a** Schematic diagram of marker-free genome editing. **b** The plasmid map of pCcPef3-126-fcy1sg2. pUC ori: origin for *Escherichia coli*, KmR: kanamycin resistance gene, AbHSP26_ter: terminator from *hsp26* gene in *Agaricus bisporus*. NLS: Nuclear localization signal, fcoCas9: Fungal and plant codon optimized Cas9 [10], CcEF3_pro: Promoter from *ef3* gene in *Coprinopsis cinerea*, CcU6_pro: U6 promoter from *C. cinerea*, CctubB_pro or ter: Promoter or terminator from tubulin gene in *C. cinerea*, *hph*: hygromycin phosphotransferase. **c** PCR amplification of *hph* fragment using primer set hph_F and hph_R. Lane M, 1-kb ladder (Nippon gene); lane P, pPHT1 (positive control); lane W, parental strain PC9 (negative control); lanes 1–6, Pef3-1–6 strains. Hyg^R: hygromycin B resistant, Hyg^S: hygromycin B sensitive

Table 3 Results of the transient transformation using *hph*-based plasmid

Plasmid	Number of strains	5-FC resistant	Hyg sensitive	Loss of *hph*
pPHT1	46	N/A	3	3
pCcpef3-126-fcy1sg2	131	130	4	1

off-target effects by avoiding constitutive expression of the CRISPR/Cas9 machinery. This editing method is expected to be suitable for creating genetically stable mutants.

This system requires a selectable marker only for initial selection of transformants. The marker is eventually lost in some transformants after repeated transfers. Hence, it is possible to repeatedly use selection markers for further genome editing using the method described in this study. This is the most important potentially novel advantage relative to other CRISPR/Cas9 protocols, including the ribonucleoprotein (RNP)-based genome-editing system. A pre-assembled RNP complex of Cas9 protein with in vitro transcribed gRNAs is an established method for performing gene mutation/disruption in eukaryotes without the possibility of transgene integration [26, 27]. In this case, the entire set required for genome editing is introduced directly into the host cell. After its introduction, these components are expected to cause genome editing and are eventually degraded by intracellular proteases and nucleases. An RNP-dependent CRISPR/ Cas9 was successfully introduced into *P. ostreatus* in our previous research [12]. Although it is expected to be a genome-editing tool without any transgene introduction, there is no way to select RNP transformants unless the
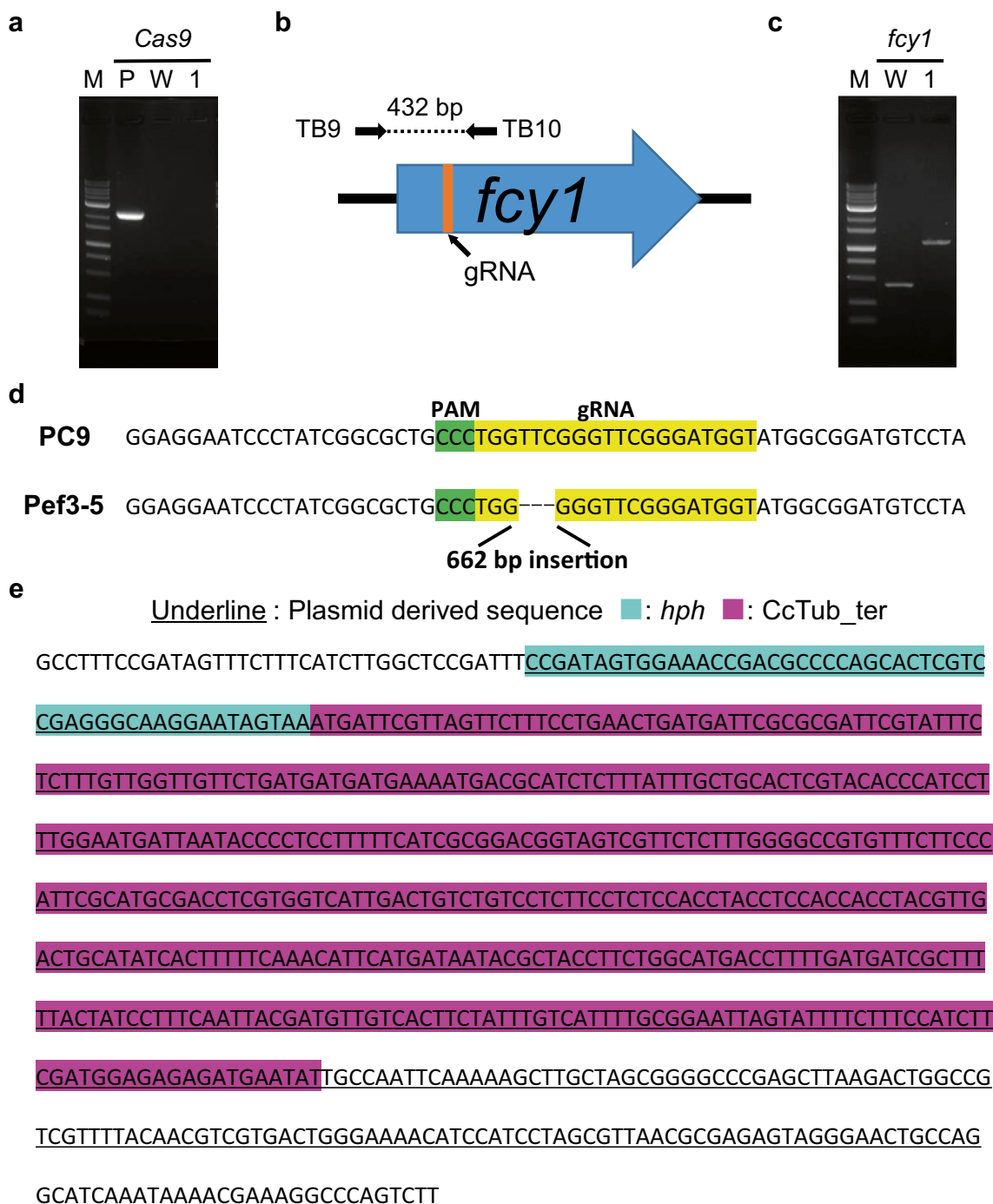
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Fig. 3 Confirmation of transient Cas9 expression and genome editing. **a** PCR amplification of *cas9* gene fragment using primer sets Cas9check_F2 and Cas9check_R2. Lane M, 1-kb ladder (Nippon gene); lane P, pCcPef3-126-fcy1sg2 (positive control); lane W, parental strain PC9 (negative control); lanes1, Pef3-5. **b** A schematic diagram of the *fcy1* loci in the PC9 parental strain with gRNA recognition sites. The dash lines indicate the region amplified by genomic PCR. Black arrows display the primer pairs used for the PCR experiments and Sanger sequencing. **c** PCR amplification of a target site of gRNA in the *fcy1* using primer set TB9 and TB10. Lane M, 1-kb ladder (Nippon gene); lane W, parental strain PC9; lanes1, Pef3-5. **d** DNA sequencing to identify mutations of *fcy1* in Pef3-5 strain. For highlights in the nucleotide sequence: yellow shades indicate gRNA, green shades indicate a protospacer adjacent motif (PAM) sequence, and dash lines indicate deletion by insertion of a plasmid sequence. **e** Insertion sequence in *fcy1* locus of Pef3-5 strain revealed by Sanger sequencing. For highlights in the nucleotide sequence: blue shades indicate a sequence derived from *hph*, magenta shades indicate a sequence derived from CcTub_ter, and under lines indicate sequence derived from pCcPef3-126-fcy1sg2

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target gene functions as a selectable marker for genome editing. Therefore, donor DNA with a selection marker to be integrated into the host genome is usually used for direct introduction of Cas9 RNP [26]. Compared to this method, our transient expression system allows initial selection and genome editing of transformants via temporal expression of Cas9, gRNA, and the selection marker, and no transgene integration can be expected by further screening.

Generally, the frequency of homologous recombination in agaricomycetes is very low [16, 28], which may be due to high NHEJ activity. To effectively carry out gene targeting using conventional methods in agaricomycetes, isolation of a strain lacking the NHEJ pathway is essential. However, it is not easy to isolate such strains when ectopic incorporation of the introduced DNA occurs randomly. With our new protocol for transient CRISPR/Cas9-directed genome editing, multiple gene disruptions can be achieved in wild-type strains without isolating NHEJ-deficient strains. Therefore, this method is useful for editing multiple genes in many fungal species. Furthermore, using knock-in constructs, the variation of genome editing may be expanded largely using this method. To improve efficiency and reduce unexpected transgene integration in the chromosome, further technical improvements are being investigated.

Conclusion

In this study, transient expression of *hph* was used to select the transformants. Then, marker-free genome editing was achieved by the transient expression of Cas9, gRNA, and *hph*. Our new genome-editing system is expected to acquire fewer off-target effects by CRISPR/Cas9 and allow the repeated use of the selection marker to investigate the functions of multi-copy genes such as ligninolytic enzyme genes in the future.

Abbreviations

CRISPR: Clustered regularly interspaced short palindromic repeat; Cas9: CRISPR-associated protein 9; gRNA: Guide RNA; Hyg: Hygromycin B; *hph*: Hygromycin phosphotransferase; VP: Versatile peroxidase; MnP: Manganese peroxidase; *fcy1*: Cytosine deaminase; 5-FC: 5-Fluorocytosine; RNP: Ribonucleoprotein; NHEJ: Non-homologous end joining; YMG: Yeast and malt extract with glucose; PCR: Polymerase chain reaction; PAM: Protospacer adjacent motif.

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Author contributions

MK, TI and YH conceived and designed the study. HU, DK, MK, and MH performed the experiments. HU, DK and MK drafted the manuscript. HU, DK, and MK performed analyses. TN, MS, KI, TS, TI, and YH provided the editorial suggestions and revisions. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

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