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Absence of a gene encoding cytosine deaminase in the genome of the agaricomycete Coprinopsis cinerea enables simple marker recycling through 5-fluorocytosine counter-selection

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Running title: Marker recycling in Coprinopsis cinerea
Abstract

*Coprinopsis cinerea* is a model species for molecular genetics studies of sexual development in agaricomycetes or homobasidiomycetes. Recently, efficient gene targeting was established in this fungus by generating *Cc.ku70* or *Cc.lig4* disruptants. To determine the molecular mechanisms underlying sexual development, which involves many genes, generating multiple gene disruptants is required. However, the number of transformation markers available for *C. cinerea* is limited. This problem would be solved by establishing marker recycling. In this study, we found that *C. cinerea* lacks a gene encoding a homolog of *Saccharomyces cerevisiae* cytosine deaminase (Fcy1p) in its genome, which is present in many other fungi. We also observed that *C. cinerea* is resistant to 5-fluorocytosine. Based on these findings, we established a simple marker recycling method in this fungus using 5-fluorocytosine counter-selection after heterologous expression of FCY1 derived from *Pleurotus ostreatus*, together with the hygromycin resistance gene. This study proposes a simple genetic manipulation system that can be performed using wild-type strains of several fungi that lack a gene homologous to *S. cerevisiae FCY1* in their genomes.
Introduction

The inky cap mushroom *Coprinopsis cinerea* has been used for classical and molecular genetics studies of fungal multicellular development, fruiting-body or mushroom formation, for many years because it completes its life cycle in 2 weeks and produces abundant oidia, which are uninucleate asexual spores (for reviews, see Kües 2000; Kamada 2002). Its easy and reliable genetic transformation system (Granado *et al.* 1997; Cummings *et al.* 1999; Ito *et al.* 2004), useful tools for genetic analysis (Zolan *et al.* 1992; Muraguchi *et al.* 2003) and carefully assembled genomic sequence (Stajich *et al.* 2010) also make it very easy to conduct molecular genetic studies in *C. cinerea* through forward genetics. However, gene targeting through homologous recombination to disrupt or modify genes of interest is challenging in this fungus, because the frequency of homologous recombination is very low, almost 0%. This has been a serious bottleneck in *C. cinerea* studies. Recently, Nakazawa *et al.* (2011) established high-frequency gene targeting in *C. cinerea* by generating strains in which *Cc.ku70* or *Cc.lig4* are disrupted.

Efficient gene targeting in other agaricomycetes, such as *Schizophyllum commune* and *Pleurotus ostreatus*, has also been established (de Jong *et al.* 2010; Salame *et al.* 2012). However, it remains difficult to generate strains in which multiple genes are modified or disrupted, because the number of transformation markers available in these fungi is limited. Therefore, a marker recycling system should be developed in agaricomycetes. In various filamentous fungi, marker recycling has been established by 5-fluoroorotic acid (5-FOA) counter-selection, which is carried out after auxotrophic complementary transformation of *pyrG* mutants (Boeke *et al.* 1984; Yoon *et al.* 2011; Nakazawa *et al.* 2013). However, mutations in
pyrG generally cause uridine/uracil auxotrophy. Even when pyrG mutants are cultured on complete media, such as yeast and malt extract with glucose (YMG) medium, it is often necessary to supply a large amount of uridine for their hyphal growth, which might cause unexpected physiological and biochemical effect(s) in the fungus and make it difficult to examine the precise effects of gene disruption on the phenotype of interest. Therefore, it is desirable to establish a different marker recycling system.

The FCYI gene, which encodes cytosine deaminase, has been used frequently for 5-fluorocytosine (5-FC) counter-selection in yeasts (Jund et al. 1970; Erbs et al. 1997). As far as we know, mutations in FCYI have never been reported to cause auxotrophy in yeast. Furthermore, in silico analysis suggested that wild-type C. cinerea lacks a gene homologous to yeast FCYI in its genome, suggesting that generating an fcyI mutant sensitive to 5-FC, which is a prerequisite for 5-FC counter-selection, would not be required. Therefore, this counter-selection system would be more suitable in this fungus.

Materials and Methods

Strains, culture conditions and genetic techniques of C. cinerea and P. ostreatus

The C. cinerea and P. ostreatus strains used in this study are listed in Table 1. YMG medium (Rao and Niederpruem 1969) solidified with 2% (w/v) agar in 4- or 9-cm Petri dishes was used for routine cultures. Cultures were maintained at 28°C under continuous darkness. For fruiting and oidia production, C. cinerea strains were cultured on YMG slants or YMG agar plates at 28°C under a 12-h light/12-h dark cycle. Crosses were performed as described by Inada et al. (2001). In this study, transformation was performed as described by Nakazawa et al.
(2010), using protoplasts prepared from mycelial cells.

**Construction of plasmids to express PoFCY1 in C. cinerea**

To express *P. ostreatus* FCY1 (PoFCY1) under the control of the *C. cinerea* $\beta_1$-tub promoter, which has been the most frequently used promoter for heterologous expression of proteins in this fungus (Cummings *et al.* 1999; Nakazawa *et al.* 2009; Muraguchi *et al.* 2011), we performed inverse polymerase chain reaction (PCR) using pPHT1 (Cummings *et al.* 1999) as a template and the primer set TN86-TN88 to yield linear DNA in which *hph*, the gene encoding hygromycin phosphotransferase (conferring resistance to hygromycin B), was deleted from pPHT1. The cDNA fragment of *Pofcy1* (from the predicted translation start site to the stop site shown in the genome web site described in the Results and discussion) was also amplified by reverse-transcription (RT)-PCR. In this study, total RNAs from *P. ostreatus* PC9 were reverse-transcribed using SuperScript III Reverse Transcriptase (Life Technologies, CA, USA), followed by a conventional PCR reaction using the primer set TN84-TN85, to amplify the cDNA fragment of *Pofcy1*. The resulting two DNA fragments were fused using a Geneart Seamless Cloning and Assembly kit (Life Technologies, CA, USA). We designated the resulting plasmid pTN2000. To construct a plasmid to express HPH fused with PoFCY1, we performed inverse PCR using pPHT1 as a template and the primer set TN87-TN88. The amplified linear DNA was fused with the cDNA fragment of *Pofcy1* prepared above using the Geneart Seamless Cloning and Assembly kit: the resulting plasmid was designated pTN2001.

To amplify the genomic fragment containing a putative open reading frame (ORF) of *Pofcy1*, together with its 5′-upstream and 3′-downstream sequences, we performed PCR with
genomic DNAs from strain PC9 as a template and the primer set TN101-TN102. The amplified
genomic fragment was cloned into pBluescript II KS+ digested with EcoRV. The resulting
plasmid was digested with HindIII and EcoRI, followed by reaction with the Klenow Fragment
(Takara Bio, Shiga, Japan). This DNA fragment, containing the Pofcy1 gene, was inserted into
pPHT1 digested with EcoRV to yield plasmid pTN2002, which contained the expression
cassette for HPH from plasmid pPHT1 and the Pofcy1 gene from strain PC9. Next, pTN2000
was digested with EcoRI to obtain the expression cassette for PoFCY1 under the control of the
C. cinerea β1-tub promoter. The obtained DNA fragment was treated with the Klenow Fragment
and inserted into pPHT1 digested with EcoRV to yield plasmid pTN2003, which contained
expression cassettes for HPH and PoFCY1 from pPHT1 and pTN2000, respectively. The C.
cinerea β1-tub promoter from plasmid pPHT1 and the genomic fragment containing the
putative ORF of Pofcy1 and its 3′-downstream region from P. ostreatus strain PC9 were
amplified by PCR using primer sets TN103-TN86 and TN84-TN102, respectively. They were
then fused by overlap extension PCR. The resulting expression cassette for PoFCY1 was
inserted into pPHT1 digested with EcoRV after treatment with T4 Polynucleotide Kinase
(Takara Bio, Shiga, Japan) to yield plasmid pTN2004.

A DNA fragment containing the 3′-downstream sequence of Pofcy1 was amplified by
PCR using the primer set TN114-TN115 (the template was pTN2004). The resulting DNA
fragment (351 bp) and plasmid pTN2004 were both digested with BamHI and HindIII, and then
fused to yield plasmid pTN2005, which contained two direct repeat sequences (the
3′-downstream sequence of Pofcy1) that were there to mediate intramolecular homologous
recombination to remove the expression cassettes for HPH and PoFCY1 contained in plasmid
Construction of the Cc.mpr1-disrupting plasmid

A genomic fragment (Scaffold 4: 373685–377348), amplified using the genomic DNA from ku3-24 as a template and primer set TN120-TN123, was cloned into pBluescript II KS+ digested with EcoRV. Inverse PCR was performed with the resulting plasmid as a template and the primer set TN121-TN122. A DNA fragment containing the expression cassettes for HPH and PoFCY1 was also amplified by PCR using pTN2005 as the template and the primer set M13F-M13R. The resulting two DNA fragments were fused using the Geneart Seamless Cloning and Assembly kit to yield a plasmid containing the Cc.mpr1-disruption cassette. The protein ID used to search and obtain sequence data for Cc.mpr1 (and the protein encoded by it) from the genome database described in the Results and discussion was 469410.

Colony PCR and genomic PCR

We carried out colony PCR as described by Nakazawa et al. (2011) for rapid screening for gene disruptants and strains from which markers hph and PoFCY1 were removed, except that the EmeraldAmp MAX PCR Master Mix (Takara Bio, Shiga, Japan) was used in this study. After colony PCR, we then performed genomic PCR. Genomic DNAs were extracted as described by Zolan and Pukkila (1986) and Muraguchi et al. (2003).

5-FC counter-selection
Strain mpr-3 was cultured on a YMG agar plate (9-cm Petri dishes) under a 12-h light/12-h dark cycle for 9 days at 28°C. Oidia (10⁷) harvested from mpr-3 were spread onto a YMG agar plate containing 0.1% (w/v) 5-FC. After 4–6 days, 5-FC resistant colonies appeared on the agar medium. They were transferred onto a YMG agar plate containing 0.1% (w/v) 5-FC only once for nuclear purification. After this passage, each of the obtained clones, rev#2–7, was inoculated onto YMG agar medium containing 100 µg/ml (w/v) hygromycin-B to examine whether they were sensitive or resistant to hygromycin-B. DNA analyses by colony PCR and genomic PCR was then carried out as above.

Results and Discussion

C. cinerea wild-type strains exhibited resistance to 5-FC

We found that C. cinerea does not possess a gene encoding a homolog of cytosine deaminase in its genome (http://genome.jgi.doe.gov/Copci_AmutBmut1/Copci_AmutBmut1.home.html), whereas P. ostreatus does (protein ID 89004 In: JGI http://genome.jgi-psf.org/PleosPC9_1/PleosPC9_1.home.html, identity = 60% (90/150), E value = 9e-61). Two C. cinerea wild-type strains, 326 and KF3#2, and P. ostreatus strain PC9 were assayed for sensitivity or resistance to 5-FC. The two C. cinerea strains were resistant to 5-FC, whereas P. ostreatus strain PC9 was sensitive (Fig. 1), which was consistent with the presence or absence of a gene homologous to yeast FCY1. This finding prompted us to examine whether heterologous expression of P. ostreatus FCY1, PoFCY1, confers sensitivity to 5-FC on C. cinerea.
Expressing PoFCY1 in C. cinerea strain 326 conferred sensitivity to 5-FC

Plasmid pTN2000, carrying the expression cassette for PoFCY1, was introduced into C. cinerea strain 326. pTN2000 was co-transformed with pPHT1 carrying the transformation marker hph (Cummings et al. 1999). Eight out of 44 strains obtained as hygromycin-B resistant transformants did not grow on YMG agar medium containing 0.1% (w/v) 5-FC, whereas they grew on YMG agar medium without it. When strain 326 was transformed with plasmid pPHT1 only, as a control, all of the obtained hygromycin-B resistant transformants (37 strains) were resistant to 5-FC (Fig. 2A), supporting the hypothesis that heterologous expression of cytosine deaminase derived from P. ostreatus in C. cinerea conferred sensitivity to 5-FC.

Examination of constructs conferring sensitivity to 5-FC on C. cinerea

Plasmid pTN2001 (Fig. 2A) was introduced into strain 326 to express HPH fused with PoFCY1. However, the number of transformants obtained by introducing pTN2001 was lower than that obtained by introducing plasmid pPHT1, and all 18 of the obtained transformants, which were isolated as hygromycin-B resistant strains, were resistant to 5-FC (Fig. 2A). This suggested that the fusion protein lost its activity and did not function as expected. We then transformed strain 326 with plasmid pTN2002 containing the Pofcy1 gene from P. ostreatus strain PC9 (Fig. 2A and B), which resulted in only two out of the 67 hygromycin-B resistant transformants exhibiting sensitivity to 5-FC. Next, we examined plasmid pTN2003. This plasmid contains expression cassettes for HPH and PoFCY1 from pPHT1 and pTN2000, respectively, both of which are expressed under the control of the C. cinerea β1-tub promoter.
Among 21 hygromycin-B resistant transformants obtained after introducing plasmid pTN2003, 14 strains were sensitive to 5-FC (Fig. 2A and B). Furthermore, plasmid pTN2004 (Fig. 2A) was constructed as described in Materials and Methods, and was introduced into strain 326, resulting in 20 out of 33 hygromycin-B resistant transformants being sensitive to 5-FC (Fig. 2A). The fact that plasmid pTN2002 did not confer sensitivity to 5FC on strain 326 efficiently, whereas plasmids pTN2003 and pTN2004 did, suggested that the *Pofcy1* promoter derived from *P. ostreatus* strain PC9 did not function in *C. cinerea* or could not express sufficient mRNA to confer sensitivity to 5-FC on strain 326.

Based on these results, we created plasmid pTN2005 so that marker recycling through 5-FC counter-selection would be demonstrated after gene targeting experiments (see Materials and Methods and Fig. 4A).

**Targeted disruption of Cc.mpr1 encoding a putative acetyltransferase similar to Saccharomyces cerevisiae Mpr1**

We used strain ku3-24, which was newly generated in this study (Table 1), instead of 326 for efficient gene targeting experiment (Nakazawa *et al.* 2011). We also confirmed that strain ku3-24 was resistant to 5-FC like strains 326 and KF3#2 (data not shown).

We transformed *C. cinerea* strain ku3-24 with the *Cc.mpr1*-disrupting construct created as described in Materials and Methods to replace the entire ORF of *Cc.mpr1* encoding a putative acetyltransferase homologous *S. cerevisiae* Mpr1p (*siga1278b* gene for L-proline-analogue resistance) (identity = 39% (73/183), *E* value = 3e-32) (Kimura *et al.* 2002) with the construct contained in plasmid pTN2005 (Figs. 3A and 4A). Two *Cc.mpr1* disruptants,
mpr-1 and mpr-3, both of which exhibited sensitivity to 5-FC, were obtained (Fig. 3B). mpr-2 was also shown to possess nuclei in which Cc.mpr1 was disrupted (the left part of Fig. 3B). However, this strain exhibited slight resistance to 5-FC (data not shown), suggesting nuclear purification was insufficient. Genomic PCR using the primer set TN144-TN145 also suggested that mpr-2 possesses nuclei in which Cc.mpr1 was not disrupted (white arrow in the right part of Fig. 3B). Therefore, we did not consider this transformant as a Cc.mpr1 disruptant.

5-FC counter-selection to isolate a nucleus in which marker excision occurred

Oidia harvested from strain mpr-3 were subject to 5-FC counter-selection as described in Materials and Methods, resulting in the isolation of six 5-FC resistant strains, rev#2–7. Two of the six strains, rev#2 and rev#5, exhibited sensitivity to hygromycin B, suggesting that the construct used for Cc.mpr1 disruption was removed through intramolecular homologous recombination mediated by the two direct repeat sequences (thick arrows shown in Fig. 4A) in these two strains. Colony PCR was performed to examine whether marker excision had occurred (Fig. 4B). A 5.4-kb fragment was expected to be amplified from the genome in which the entire ORF of Cc.mpr1 was replaced with the construct (before marker excision/5-FC counter selection, as shown in Fig. 4A), whereas a 2.1-kb fragment was expected to be amplified from the genome in which the construct contained in pTN2005 used for Cc.mpr1 disruption was removed (after marker excision/5-FC counter selection, as shown in Fig. 4A). The results shown in Fig. 4B suggested that the construct has been successfully removed in strains rev#2 and rev#5. Both DNA fragments were amplified from strain rev#7, which suggested that this strain possessed both types of nuclei: those in which the construct was
removed and those that still retained the construct. This might indicate that the single passage used for nuclear purification was insufficient for this strain.

We then performed genomic PCR for strains rev#2, rev#5, mpr-3 and ku3-24 as a control (Fig. 4C), demonstrating that the construct contained in pTN2005 had been successfully removed in strains rev#2 and rev#5. Small amounts of 2.0- and 2.1- kb fragments, indicated by white arrows in Fig. 4C, which were expected to be amplified from the genome of strains in which the construct contained in pTN2005 used for Cc.mpr1 disruption was removed (after marker excision/5-FC counter selection as shown in Fig. 4A), were also amplified from genomic DNAs from strain mpr-3. This showed that mpr-3 contained a small number of nuclei in which intramolecular homologous recombination had occurred, and that these nuclei were purified through 5-FC counter-selection.

There are another direct repeat sequences (the $\beta$1-tub promoter used for expressions of both HPH and PoFCY1) in plasmid pTN2005, and the construct used for Cc.mpr1 disruption (Fig. 4A) Therefore, intramolecular homologous recombination mediated by these sequences might also occur. However, nuclei in which such intramolecular homologous recombination had occurred were not isolated through 5-FC counter selection in this study. This might be because the nuclei retained the expression cassette for PoFCY1, despite the occurrence of intramolecular homologous recombination, which continued to confer sensitivity to 5-FC on C. cinerea strains.

Conclusions

In this study, we presented a simple and useful marker recycling system in C. cinerea. This new genetic manipulation system, which does not cause any observable auxotrophy, could
be used as a means to create multiple gene disruptants for detailed genetic studies in this fungus.

Genome database searching suggested that some fungi, such as Agaricus bisporus, Magnaporthe oryzae and Chaetomium globosum also lack a gene encoding an Fcy1p homolog in their genomes. Therefore, marker recycling based on fcy1/5-FC counter-selection could be performed in these fungi, as well as in C. cinerea. It would also be interesting to investigate why these fungi lack a gene encoding cytosine deaminase.

Acknowledgments

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**Figure legends**

**Fig. 1.** *C. cinerea* strains exhibited resistance to 5-FC. *C. cinerea* strains 326, KF3#2 and *P. ostreatus* strain PC9 were cultured on yeast and malt extract with glucose (YMG) agar plates with and without 0.1% (w/v) 5-fluorocytosine (5-FC). Strains 326 and KF3#2 were cultured at 28°C for 4 days. Strain PC9 was cultured at 28°C for 6 days. The scale bars represent 2 cm.

**Fig. 2.** Heterologous expression of *PoFCY1* in *C. cinerea* strain 326 conferred sensitivity to 5-FC. (A) Plasmids pTN2000–pTN2004 containing different expression cassettes for *PoFCY1* were used to confer sensitivity to 5-fluorocytosine (5-FC) on *C. cinerea* strain 326, “5-FCS / total transformants” indicates the number of transformants exhibiting sensitivity to 5-FC among all of the hygromycin-B resistant transformants obtained after introducing each plasmid. Small white bars shown in plasmids pTN2002 and pTN2004 indicate predicted introns. (B) An example of conferring sensitivity to 5-FC by heterologous expression of *PoFCY1* in *C. cinerea* strain 326. White arrows indicate 5-FC-sensitive transformants obtained after introducing plasmid pTN2003.

**Fig. 3.** Targeted disruption of *Cc.mpr1*. (A) A diagram of the genomic locus of *Cc.mpr1*. Black arrows indicate the primers used for the PCR experiments in (B). (B) Genomic PCR experiments confirming *Cc.mpr1* disruption. Lane 1, the parental strain ku3-24 as a control; Lane 2, mpr-1; Lane 3, mpr-2; Lane 4, mpr-3. Lanes M on the left and right sides indicate size markers: λ.HindIII/EcoRI markers on the left and a 100 bp ladder marker (100–1000 bp) on the right.
**Fig. 4.** Isolation of a nucleus in which marker excision occurred through 5-fluorocytosine (5-FC) counter-selection, and PCR experiments confirming that it had occurred. (A) Diagram of the genomic locus of the *Cc.mpr1* gene in strain ku3-24, *Cc.mpr1* disruptants from ku3-24 (before marker excision) and strains in which the construct used for *Cc.mpr1* disruption was removed by intramolecular homologous recombination (after marker excision). Thin arrows indicate the primers used for PCR. Thick arrows indicate direct repeat sequences that mediate intramolecular homologous recombination to remove expression cassettes for HPH and *PoFCY1*. (B) Colony PCR screening for strains in which the construct used for *Cc.mpr1* disruption was removed. Lane M, λ*HindIII/EcoRI markers; #2-#7 (described as rev#2-rev#7 in the main text), 5-FC-resistant strains isolated after 5-FC counter-selection against strain mpr-3. Hyg “S” indicates a hygromycin-B sensitive strain, whereas “R” indicates a resistant strain. (C) Genomic PCR experiments confirming marker excision had occurred. Lane M, size markers (λ*HindIII* for the left side, and a 100 bp ladder marker (100–1000 bp) for the right side); Lane 1, rev#2; Lane 2, rev#5; Lane 3, mpr-3; Lane 4, ku3-24.
Wild-type *C. cinerea*

*P. ostreatus fcy1*

*fcy1 absence*  
(5-FC<sup>R</sup>)

*fcy1 presence*  
(5-FC<sup>S</sup>)

Marker excision  
(5-FC counter-selection)
Fig. 1. Nakazawa et al.

YMG   YMG + 5-FC

326

KF₃#2

PC9
Fig. 2. Nakazawa et al.
Fig. 3. Nakazawa et al.
**Fig. 4. Nakazawa et al.**

A. Schematic diagram showing the marker excision and 5-FC counter-selection process. The wild type contains the cassette (hygS 5-FCR) before marker excision, while the Cc.mpr1 disruptant contains the cassette (hygR 5-FCS) before marker excision. The marker excision results in a size difference of 687 bp (2134 bp before marker excision) and 2074 bp (2074 bp before marker excision).

B. Gel electrophoresis showing the Cc.mpr1d#3 rev disruption with sizes ranging from 6.6kb to 0.5kb.

C. Gel electrophoresis showing the TN146-M13R and M13F-TN147 disruption with sizes ranging from 5.0kb to 2.0kb.

The images and sizes are indicative of the successful marker excision and counter-selection process.
Table 1. Strains used in this study.

<table>
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<tr>
<th>Strain (species)</th>
<th>genotype/description</th>
<th>Source</th>
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<tbody>
<tr>
<td>326 (C. cinerea)</td>
<td>A43mut B43mut pab1-1</td>
<td>P.J. Pukkila</td>
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<td>PC9 (P. ostreatus)</td>
<td>A2 B1</td>
<td>Larraya et al. (1999)</td>
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<td>KF3#2 (C. cinerea)</td>
<td>A91 B91</td>
<td>Muraguchi et al. (2003)</td>
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<tr>
<td>ku3-24 (C. cinerea)</td>
<td>A43mut B43mut pab1-1 ΔCc.ku70 (FltR)</td>
<td>This study</td>
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<td>/a progeny of ku70dfltF2#92 x KF3#2</td>
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<td>Table 2. Primers used in this study.</td>
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