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

Takehito Nakazawa*, Yoichi Honda

Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

*Corresponding author: Takehito Nakazawa
Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto, Kyoto 606-8502, Japan
Tel: +81 75 753 6465
Fax: +81 75 753 6471
E-mail: tnakazaw@kais.kyoto-u.ac.jp

Keywords: basidiomycete, *Coprinopsis cinerea*, *Pleurotus ostreatus*, *FCY1*, *pyrG*, cytosine deaminase

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 *FCY1* 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 *FCY1* 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 *FCY1* in its genome, suggesting that generating an *fcy1* 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*.

111 (2010), using protoplasts prepared from mycelial cells.

112

113 *Construction of plasmids to express* Po*FCY1 in C*. cinerea

114 To express *P. ostreatus* FCY1 (*Po*FCY1) under the control of the *C. cinerea β1-tub*

115 promoter, which has been the most frequently used promoter for heterologous expression of

116 proteins in this fungus (Cummings *et al*. 1999; Nakazawa *et al*. 2009; Muraguchi *et al*. 2011),

117 we performed inverse polymerase chain reaction (PCR) using pPHT1 (Cummings *et al*. 1999)

118 as a template and the primer set TN86-TN88 to yield linear DNA in which *hph*, the gene

119 encoding hygromycin phosphotransferase (conferring resistance to hygromycin B), was deleted

120 from pPHT1. The cDNA fragment of *Pofcy1* (from the predicted translation start site to the stop

121 site shown in the genome web site described in the Results and discussion) was also amplified

122 by reverse-transcription (RT)-PCR. In this study, total RNAs from *P. ostreatus* PC9 were

123 reverse-transcribed using SuperScript III Reverse Transcriptase (Life Technologies, CA, USA),

124 followed by a conventional PCR reaction using the primer set TN84-TN85, to amplify the

125 cDNA fragment of *Pofcy1*. The resulting two DNA fragments were fused using a Geneart

126 Seamless Cloning and Assembly kit (Life Technologies, CA, USA). We designated the resulting

127 plasmid pTN2000. To construct a plasmid to express HPH fused with *Po*FCY1, we performed

128 inverse PCR using pPHT1 as a template and the primer set TN87-TN88. The amplified linear

129 DNA was fused with the cDNA fragment of *Pofcy1* prepared above using the Geneart Seamless

130 Cloning and Assembly kit: the resulting plasmid was designated pTN2001.

131 To amplify the genomic fragment containing a putative open reading frame (ORF) of

132 *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 *Eco*RV. The resulting plasmid was digested with *Hin*dIII and *Eco*RI, followed by reaction with the Klenow Fragment (Takara Bio, Shiga, Japan). This DNA fragment, containing the *Pofcy1* gene, was inserted into pPHT1 digested with *Eco*RV 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 *Eco*RI to obtain the expression cassette for *Po*FCY1 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 *Eco*RV to yield plasmid pTN2003, which contained expression cassettes for HPH and *Po*FCY1 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 *Po*FCY1 was inserted into pPHT1 digested with *Eco*RV 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 *Bam*HI and *Hin*dIII, 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 *Po*FCY1 contained in plasmid

pTN2005 (Fig. 4A).

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 *Eco*RV. 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 *Po*FCY1 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 (10^7) 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 KF_3#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, *Po*FCY1, confers sensitivity to 5-FC on *C. cinerea*.

199

200 *Expressing* Po*FCY1 in* C. cinerea *strain 326 conferred sensitivity to 5-FC*

201 Plasmid pTN2000, carrying the expression cassette for *Po*FCY1, was introduced into
202 *C. cinerea* strain 326. pTN2000 was co-transformed with pPHT1 carrying the transformation
203 marker *hph* (Cummings *et al*. 1999). Eight out of 44 strains obtained as hygromycin-B resistant
204 transformants did not grow on YMG agar medium containing 0.1% (w/v) 5-FC, whereas they
205 grew on YMG agar medium without it. When strain 326 was transformed with plasmid pPHT1
206 only, as a control, all of the obtained hygromycin-B resistant transformants (37 strains) were
207 resistant to 5-FC (Fig. 2A), supporting the hypothesis that heterologous expression of cytosine
208 deaminase derived from *P. ostreatus* in *C. cinerea* conferred sensitivity to 5-FC.

209

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

211 Plasmid pTN2001 (Fig. 2A) was introduced into strain 326 to express HPH fused
212 with *Po*FCY1. However, the number of transformants obtained by introducing pTN2001 was
213 lower than that obtained by introducing plasmid pPHT1, and all 18 of the obtained
214 transformants, which were isolated as hygromycin-B resistant strains, were resistant to 5-FC
215 (Fig. 2A). This suggested that the fusion protein lost its activity and did not function as expected.
216 We then transformed strain 326 with plasmid pTN2002 containing the *Pofcy1* gene from *P.*
217 *ostreatus* strain PC9 (Fig. 2A and B), which resulted in only two out of the 67 hygromycin-B
218 resistant transformants exhibiting sensitivity to 5-FC. Next, we examined plasmid pTN2003.
219 This plasmid contains expression cassettes for HPH and *Po*FCY1 from pPHT1 and pTN2000,
220 respectively, both of which are expressed under the control of the *C. cinerea β1-tub* promoter

221 (Fig. 2A). Among 21 hygromycin-B resistant transformants obtained after introducing plasmid
222 pTN2003, 14 strains were sensitive to 5-FC (Fig. 2A and B). Furthermore, plasmid pTN2004
223 (Fig. 2A) was constructed as described in Materials and Methods, and was introduced into strain
224 326, resulting in 20 out of 33 hygromycin-B resistant transformants being sensitive to 5-FC (Fig.
225 2A). The fact that plasmid pTN2002 did not confer sensitivity to 5FC on strain 326 efficiently,
226 whereas plasmids pTN2003 and pTN2004 did, suggested that the *Pofcy1* promoter derived from
227 *P. ostreatus* strain PC9 did not function in *C. cinerea* or could not express sufficient mRNA to
228 confer sensitivity to 5-FC on strain 326.
229 Based on these results, we created plasmid pTN2005 so that marker recycling
230 through 5-FC counter-selection would be demonstrated after gene targeting experiments (see
231 Materials and Methods and Fig. 4A).
232
233 *Targeted disruption of Cc.mpr1 encoding a putative acetyltransferase similar to* Saccharomyces
234 cerevisiae *Mpr1*
235 We used strain ku3-24, which was newly generated in this study (Table 1), instead of
236 326 for efficient gene targeting experiment (Nakazawa *et al.* 2011). We also confirmed that
237 strain ku3-24 was resistant to 5-FC like strains 326 and KF_3#2 (data not shown).
238 We transformed *C. cinerea* strain ku3-24 with the *Cc.mpr1*-disrupting construct
239 created as described in Materials and Methods to replace the entire ORF of *Cc.mpr1* encoding a
240 putative acetyltransferase homologous *S. cerevisiae* Mpr1p (sigma1278b gene for
241 L-proline-analogue resistance) (identity = 39% (73/183), *E* value = 3e-32) (Kimura *et al*. 2002)
242 with the construct contained in plasmid pTN2005 (Figs. 3A and 4A). Two *Cc.mpr1* disruptants,

243 mpr-1 and mpr-3, both of which exhibited sensitivity to 5-FC, were obtained (Fig. 3B). mpr-2
244 was also shown to possess nuclei in which *Cc.mpr1* was disrupted (the left part of Fig. 3B).
245 However, this strain exhibited slight resistance to 5-FC (data not shown), suggesting nuclear
246 purification was insufficient. Genomic PCR using the primer set TN144-TN145 also suggested
247 that mpr-2 possesses nuclei in which *Cc.mpr1* was not disrupted (white arrow in the right part of
248 Fig. 3B). Therefore, we did not consider this transformant as a *Cc.mpr1* disruptant.
249
250 *5-FC counter-selection to isolate a nucleus in which marker excision occurred*
251 Oidia harvested from strain mpr-3 were subject to 5-FC counter-selection as
252 described in Materials and Methods, resulting in the isolation of six 5-FC resistant strains,
253 rev#2–7. Two of the six strains, rev#2 and rev#5, exhibited sensitivity to hygromycin B,
254 suggesting that the construct used for *Cc.mpr1* disruption was removed through intramolecular
255 homologous recombination mediated by the two direct repeat sequences (thick arrows shown in
256 Fig. 4A) in these two strains. Colony PCR was performed to examine whether marker excision
257 had occurred (Fig. 4B). A 5.4-kb fragment was expected to be amplified from the genome in
258 which the entire ORF of *Cc.mpr1* was replaced with the construct (before marker excision/5-FC
259 counter selection, as shown in Fig. 4A), whereas a 2.1-kb fragment was expected to be
260 amplified from the genome in which the construct contained in pTN2005 used for *Cc.mpr1*
261 disruption was removed (after marker excision/5-FC counter selection, as shown in Fig. 4A).
262 The results shown in Fig. 4B suggested that the construct has been successfully removed in
263 strains rev#2 and rev#5. Both DNA fragments were amplified from strain rev#7, which
264 suggested that this strain possessed both types of nuclei: those in which the construct was

265 removed and those that still retained the construct. This might indicate that the single passage
266 used for nuclear purification was insufficient for this strain.
267 We then performed genomic PCR for strains rev#2, rev#5, mpr-3 and ku3-24 as a
268 control (Fig. 4C), demonstrating that the construct contained in pTN2005 had been successfully
269 removed in strains rev#2 and rev#5. Small amounts of 2.0- and 2.1- kb fragments, indicated by
270 white arrows in Fig. 4C, which were expected to be amplified from the genome of strains in
271 which the construct contained in pTN2005 used for *Cc.mpr1* disruption was removed (after
272 marker excision/5-FC counter selection as shown in Fig. 4A), were also amplified from
273 genomic DNAs from strain mpr-3. This showed that mpr-3 contained a small number of nuclei
274 in which intramolecular homologous recombination had occurred, and that these nuclei were
275 purified through 5-FC counter-selection.
276 There are another direct repeat sequences (the *β1-tub* promoter used for expressions
277 of both HPH and *Po*FCY1) in plasmid pTN2005, and the construct used for *Cc.mpr1* disruption
278 (Fig. 4A) Therefore, intramolecular homologous recombination mediated by these sequences
279 might also occur. However, nuclei in which such intramolecular homologous recombination had
280 occurred were not isolated through 5-FC counter selection in this study. This might be because
281 the nuclei retained the expression cassette for *Po*FCY1, despite the occurrence of intramolecular
282 homologous recombination, which continued to confer sensitivity to 5-FC on *C. cinerea* strains.
283
284 **Conclusions**
285 In this study, we presented a simple and useful marker recycling system in *C. cinerea*.
286 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

This work was supported by the Institute for Fermentation, Osaka [(no grant number) to T.N.] and the JSPS Grant-in-Aid for Exploratory Research [grant number 256601370 to Y.H.].

We thank Prof. Takashi Kamada for providing advices on preparing this manuscript, Prof. Antonio Gerardo Pisabarro for providing *P. ostreatus* strain PC9 and Dr. Hajime Muraguchi for sending plasmid pPHT1 and *C. cinerea* strains 326, KF_3#2 and ku70dfltF$_2$#92.

References

Boeke JD, LaCroute F, Fink GR. A positive selection for mutants lacking orotidine-5’-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* 1984;**197**:345–6.

Cummings WJ, Celerin M, Crodian J *et al*. Insertional mutagenesis in *Coprinus cinereus*: use of a dominant selectable marker to generate tagged, sporulation-defective mutants. *Curr Genet* 1999;**36**:371–82.

de Jong JF, Ohm RA, de Bekker C *et al*. Inactivation of *ku80* in the mushroom-forming

309 fungus *Schizophyllum commune* increases the relative incidence of homologous
310 recombination. *FEMS Microbiol Lett* 2010;**310**:91–5.
311 Erbs P, Exinger F, Jund R. Characterization of the *Saccharomyces cerevisiae*
312 *FCY1* gene encoding cytosine deaminase and its homologue *FCA1* of *Candida*
313 *albicans*. *Curr Genet* 1997;**31**:1–6.
314 Granado JD, Kertesz-Chaloupkovà K, Aebi M *et al*. Restriction enzyme-mediated DNA
315 integration in *Coprinus cinereus*. *Mol Gen Genet* 1997;**256**:28–36.
316 Inada K, Morimoto Y, Arima T *et al*. The *clp1* gene of the mushroom *Coprinus*
317 *cinereus* is essential for *A*-regulated sexual development.
318 *Genetics* 2001;**157**:133–40.
319 Ito Y, Muraguchi H, Seshime Y *et al*. Flutolanil and carboxin resistance in *Coprinus*
320 *cinereus* conferred by a mutation in the cytochrome b_{560} subunit of succinate
321 dehydrogenase complex (Complex II). *Mol Gen Genet* 2004;**272**:328–35.
322 Jund R, Lacroute F. Genetic and physiological aspects of resistance to
323 5-fluoropyrimidines in *Saccharomyces cerevisiae*. *J Bacteriol* 1970;**102**:607–15.
324 Kamada T. Molecular genetics of sexual development in the mushroom *Coprinus*
325 *cinereus*. *BioEssays* 2002;**24**:449–59.
326 Kimura Y, Nakamori S, Takagi H. Polymorphism of the *MPR1* gene required for toxic
327 proline analogue resistance in the *Saccharomyces cerevisiae* complex species.
328 *Yeast* 2000;**19**:1437–45.
329 Kües U. Life history and developmental processes in the basidiomycete *Coprinus*
330 *cinereus*. *Microbiol Mol Biol Rev* 2000;**64**:316–53.

Larraya LM, Pérez G, Peñas MM *et al*. Molecular karyotype of the white rot fungus *Pleurotus ostreatus*. *Appl Environ Microbiol* 1999;**65**:3413–7.

Muraguchi H, Ito Y, Kamada T *et al*. A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymeric DNAs and restriction fragment length polymorphisms. *Fungal Genet Biol* 2003;**40**:93–102.

Muraguchi H, Kondoh M, Ito Y *et al*. Molecular breeding of a novel *Coprinopsis cinerea* strain possessing a heterologous laccase gene, *lccK*, driven by a constitutive promoter. *Mycoscience* 2011;**52**:431–5.

Nakazawa T, Kaneko S, Murata H *et al*. The homologue of *Lentinula edodes ctg1*, a target for CDC5 and its interacting partner CIPB, from *Coprinopsis cinerea* is involved in fruiting-body morphogenesis of *C. cinerea*. *Mycoscience* 2011;**50**:331-42.

Nakazawa T, Tatsuta Y, Fujita T *et al*. Mutations in the *Cc.rmt1* gene encoding a putative protein arginine methyltransferase alter developmental programs in the basidiomycete *Coprinopsis cinerea*. *Curr Genet* 2010;**56**:361–7.

Nakazawa T, Ando Y, Kitaaki K *et al*. Efficient gene targeting in *Δ Cc.ku70* or *Δ Cc.lig4* mutants of the agaricomycete *Coprinopsis cinerea*. *Fungal Genet Biol* 2011;**48**:939–46.

Nakazawa T, Ishiuchi K, Sato M *et al*. Targeted disruption of transcriptional regulators in *Chaetomium globosum* activates biosynthetic pathways and reveals transcriptional regulator-like behavior of aureonitol. *J Am Chem Soc* 2013;**135**:13446–55.

Rao PS, Niederpruem DJ. Carbohydrate metabolism during morphogenesis of

Coprinus lagopus (sensu Buller). *J Bacteriol* 1969;**100**:1222–8.

Salame TM, Knop D, Tal D *et al.* Predominance of a versatile-peroxidase-encoding gene, *mnp4*, as demonstrated by gene replacement via a gene targeting system for *Pleurotus ostreatus*. *Appl Eviron Microbiol* 2012;**78**:5341–52.

Stajich JE, Wilke SK, Ahrén D *et al*. Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). *Proc Natl Acad Sci USA* 2010;**107**:11889–94.

Yoon J, Maruyama J, Kitamoto K. Disruption of ten protease genes in the filaments fungus *Aspergillus oryzae* highly improves production of heterologous proteins. *Appl Microbiol Biotechnol* 2011;**89**:747–59.

Zolan ME, Pukkila PJ. Inheritance of DNA methylation in *Coprinus cinereus*. *Mol Cell Biol* 1986;**6**:195–200.

Zolan ME, Crittenden J, Heyler NK *et al.* Efficient isolation and mapping of *rad* genes of the fungus *Coprinus cinereus* using chromosome-specific libraries. *Nucl Acids Res* 1992;**20**:3993–9.

375 **Figure legends**

376 **Fig. 1.** *C. cinerea* strains exhibited resistance to 5-FC. *C. cinerea* strains 326, KF_3#2 and *P.*
377 *ostreatus* strain PC9 were cultured on yeast and malt extract with glucose (YMG) agar plates
378 with and without 0.1% (w/v) 5-fluorocytosine (5-FC). Strains 326 and KF_3#2 were cultured at
379 28°C for 4 days. Strain PC9 was cultured at 28°C for 6 days. The scale bars represent 2 cm.
380
381 **Fig. 2.** Heterologous expression of *Po*FCY1 in *C. cinerea* strain 326 conferred sensitivity to
382 5-FC. (A) Plasmids pTN2000–pTN2004 containing different expression cassettes for *Po*FCY1
383 were used to confer sensitivity to 5-fluorocytosine (5-FC) on *C. cinerea* strain 326, "5-FC^S /
384 total transformants" indicates the number of transformants exhibiting sensitivity to 5-FC among
385 all of the hygromycin-B resistant transformants obtained after introducing each plasmid. Small
386 white bars shown in plasmids pTN2002 and pTN2004 indicate predicted introns. (B) An
387 example of conferring sensitivity to 5-FC by heterologous expression of *Po*FCY1 in *C. cinerea*
388 strain 326. White arrows indicate 5-FC-sensitive transformants obtained after introducing
389 plasmid pTN2003.
390
391 **Fig. 3.** Targeted disruption of *Cc.mpr1*. (A) A diagram of the genomic locus of *Cc.mpr1*. Black
392 arrows indicate the primers used for the PCR experiments in (B). (B) Genomic PCR
393 experiments confirming *Cc.mpr1* disruption. Lane 1, the parental strain ku3-24 as a control;
394 Lane 2, mpr-1; Lane 3, mpr-2; Lane 4, mpr-3. Lanes M on the left and right sides indicate size
395 markers: λ*Hin*dIII/*Eco*RI markers on the left and a 100 bp ladder marker (100–1000 bp) on the
396 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 *Po*FCY1. (B) Colony PCR screening for strains in which the construct used for *Cc.mpr1* disruption was removed. Lane M, λ*Hin*dIII/*Eco*RI 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*Hin*dIII 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*

fcy1 absence

(5-FC^{R})

marker excision

(5-FC countere-selection)

fcy1 presence

(5-FC^{S})

P. ostreatus fcy1

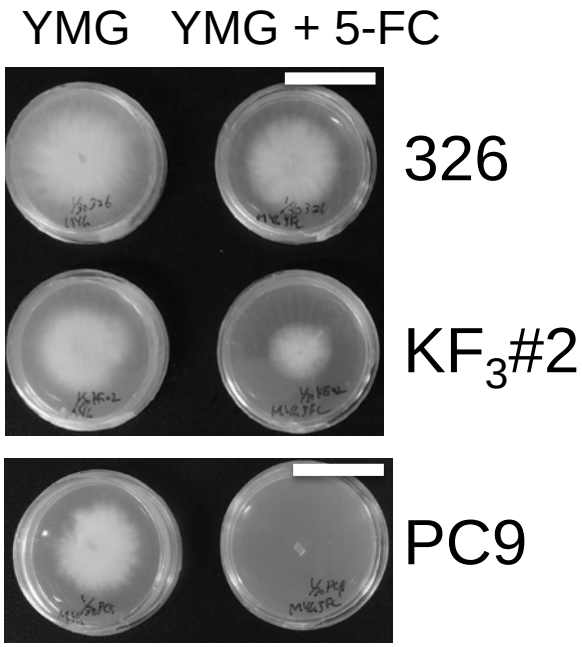
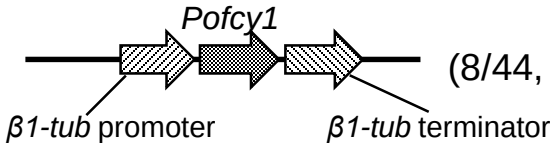


Fig. 1. Nakazawa et al.

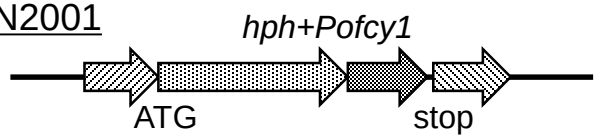
A

pTN2000 (5-FC^S / total transformants)



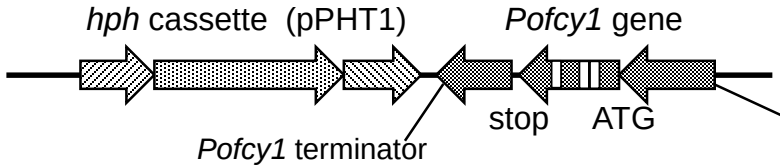
(8/44, 18%) pPHT1 only (0/37, 0%)

pTN2001



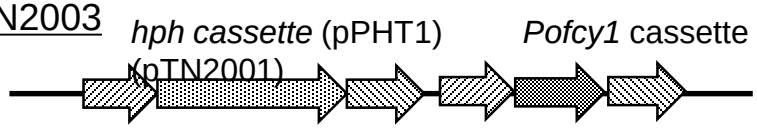
(0/18, 0%)

pTN2002



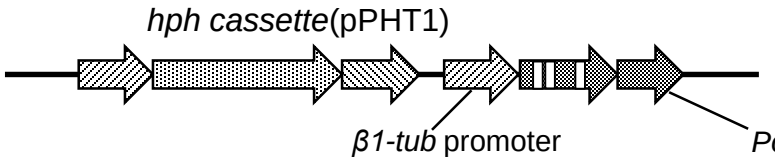
(2/67, 3%)

pTN2003



(14/21, 67%)

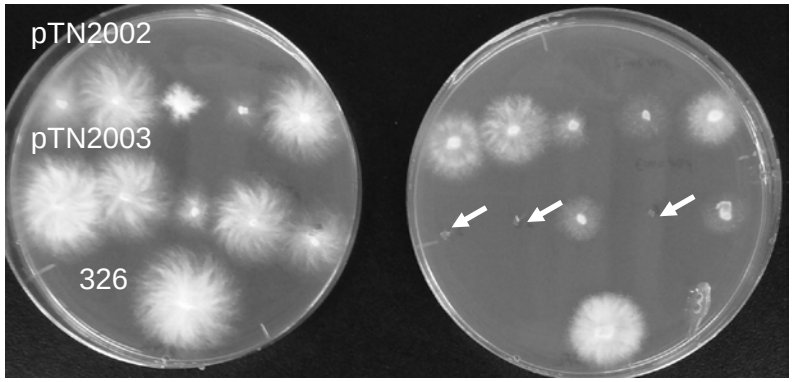
pTN2004



(20/33, 61%)

1 kb

B



YMG YMG + 5-FC

Fig. 2. Nakazawa et al.

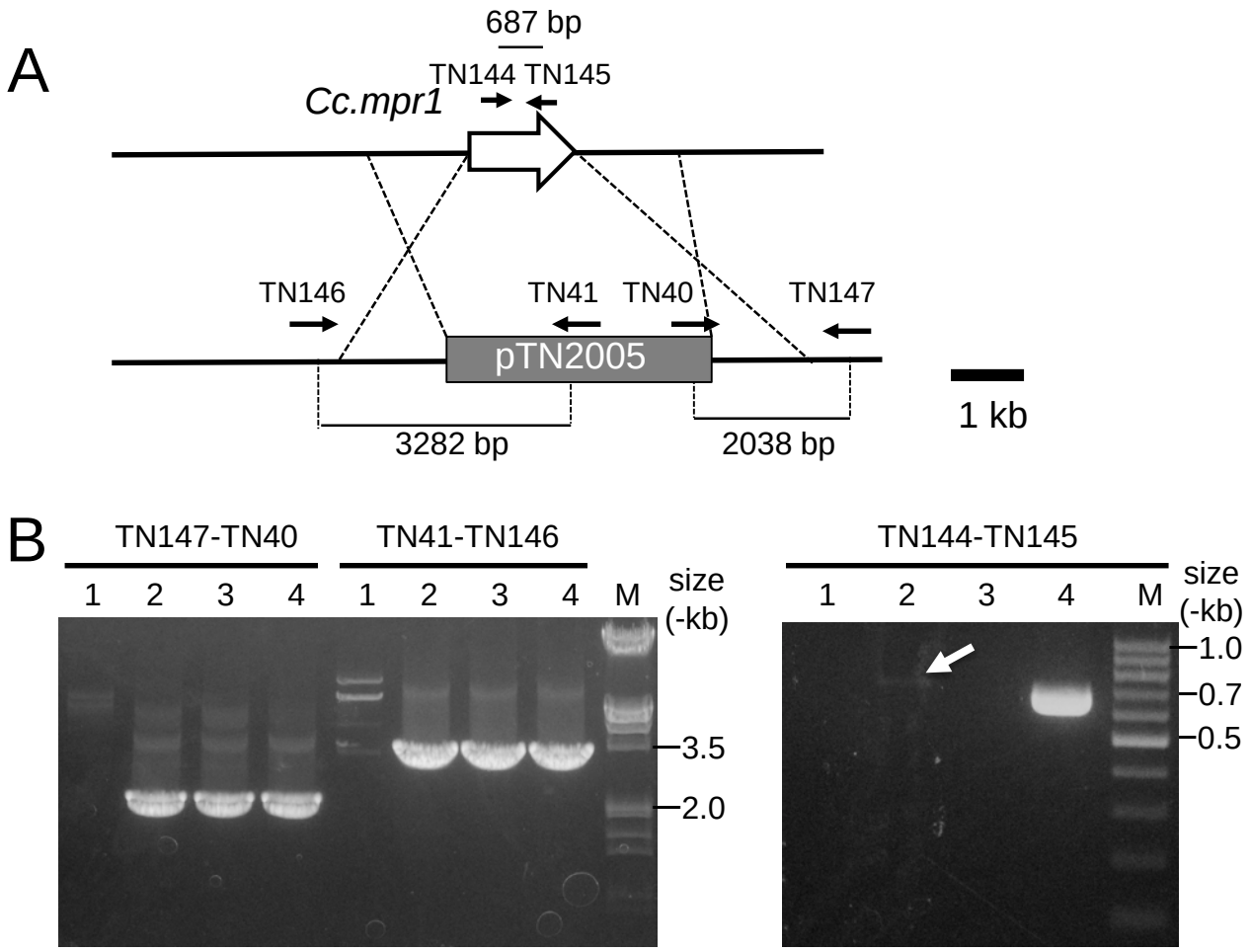


Fig. 3. Nakazawa et al.

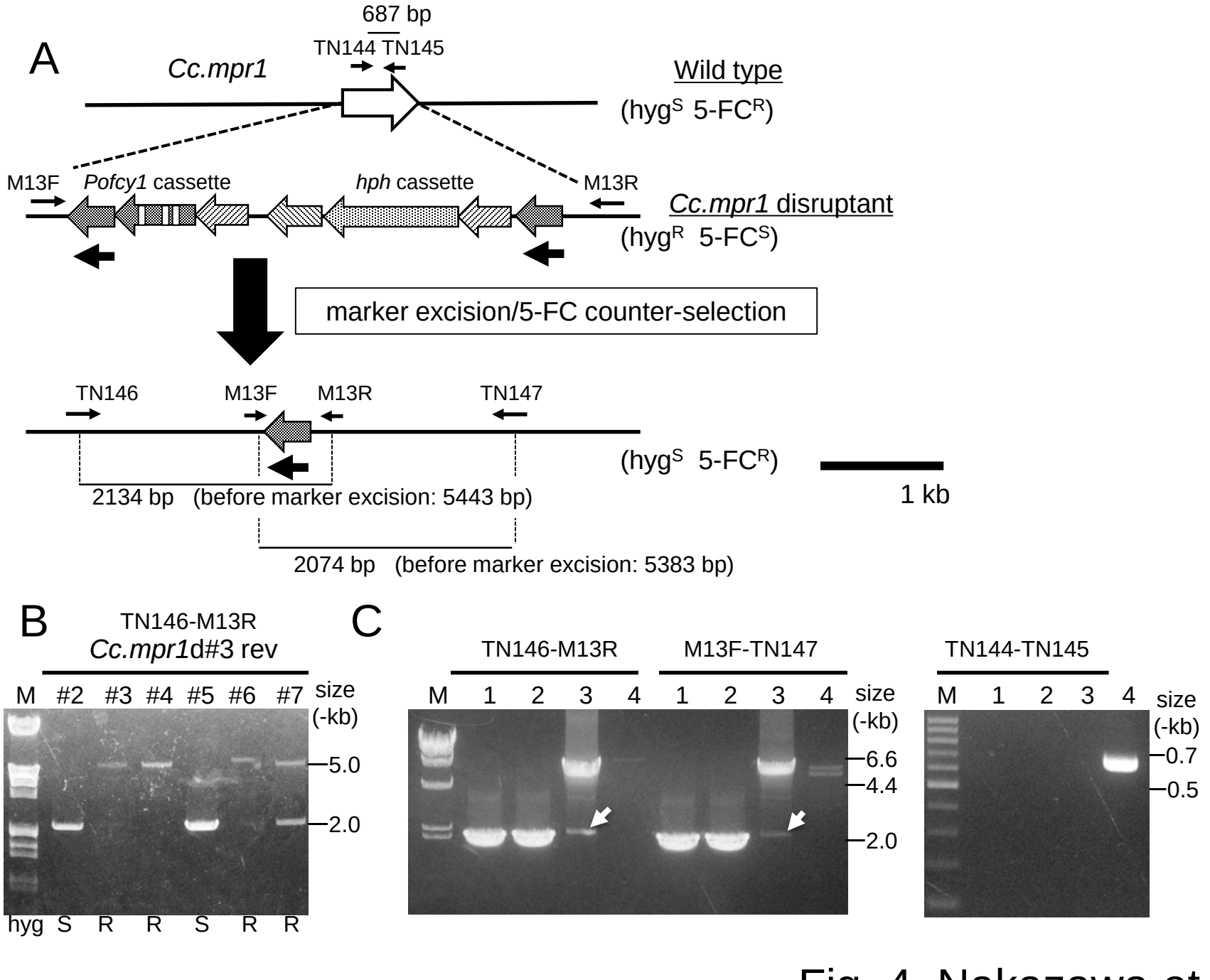


Fig. 4. Nakazawa et al.

Table 1. Strains used in this study.

Strain (species)	genotype/descryiption	Source
326 (*C. cinerea*)	*A43mut B43mut pab1-1*	P.J. Pukkila
PC9 (*P. ostreatus*)	*A2 B1*	Larraya *et al.* (1999)
KF_3#2 (*C. cinerea*)	*A91 B91*	Muraguchi *et al.* (2003)
ku3-24 (*C. cinerea*)	*A43mut B43mut pab1-1* Δ*Cc.ku70* (Flt^R) /a progeny of ku70dfltF_2#92 x KF_3#2	This study

Table 2. Primers used in this study.

TN40	ACCCTTTCCCCCAAAATTTGGAAGC
TN41	ACCTTCTGGCATGACCTTTTGATGATCGC
TN84	ATGGAAAGCGCAGATCAACTGGG
TN85	CTAGTACATCTCTCCGATGTCTTCATACC
TN86	ATCTGCGCTTTCCATGCTGGGAACGCGAGGTCAGC
TN87	ATCTGCGCTTTCCATTTCCTTTGCCCTCGGACGAG
TN88	GGAGAGATGTACTAGATGATTCGTTAGTTCTTTCC
TN101	ACGCCTCTGTCCCTTGTCCTC
TN102	ATCTCTCACCAGAACGAGGACTGC
TN103	TTCATTTAAACGGCTTCACGGGC
TN114	TATTGCGGGTGTAAGCTTAGAACGGCGG
TN115	AGGACTGGATCCAATGGTGGAACATATGAC
TN120	TCAAACTTGATCTCTCTCCCGTC
TN121	ACGTCGTGACTGGGAGGCCAAACTCGGTTCCTGTG
TN122	CCTGTGTGAAATTGTTGCCAAGACCAAAAGTGGGC
TN123	TAAGGACCGAAGAATGACCTGCTCG
TN144	TGTGCACCATCTCACGCTTCAG
TN145	TCGGCCTGCACCCGGAATTAAC
TN146	TTCTACTGTTATGCGGTTCTCTTTCCCC
TN147	ATTTTTTGGGGCCCAGCAATGC
M13F	TCCCAGTCACGACGTTGTAAAACGACGG
M13R	ACAATTTCACACAGGAAACAGCTATGACC