Biotechnol Lett (2008) 30:1811–1817
DOI 10.1007/s10529-008-9763-9

ORIGINAL RESEARCH PAPER

Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (Δ*ligD*) in *Aspergillus oryzae*

Jun-Ichi Maruyama · Katsuhiko Kitamoto

Received: 3 April 2008 / Revised: 16 May 2008 / Accepted: 30 May 2008 / Published online: 24 June 2008

Abstract Previously we reported that double disruption of the proteinase genes (*tppA* and *pepE*) improved heterologous protein production by *Aspergillus oryzae* (Jin et al. Appl Microbiol Biotechnol 76:1059–1068, 2007). Since *A. oryzae* has 134 protease genes, the number of auxotrophy in a single host is limited for multiple disruptions of many protease genes. In order to rapidly perform multiple gene disruptions in *A. oryzae*, we generated the marker recycling system in highly efficient gene-targeting background. *A. oryzae ligD* gene homologous to *Neurospora crassa mus-53* gene involved in nonhomologous chromosomal integration was disrupted, followed by disruption of the *pyrG* gene for uridine/uracil auxotroph. We further performed successive rounds of gene disruption (*tppA* and *pepE*) by the *pyrG* marker with high gene-targeting efficiency allowed by the Δ*ligD* background. After each disruption process the *pyrG* marker was excised by the direct repeats consisting of ~300 bp upstream flanking region of the target gene, resulting in no residual ectopic/foreign DNA fragments in the genome. Consequently, we succeeded to breed the double proteinase gene disruptant (Δ*tppA* Δ*pepE*) applicable to further sequential gene disruptions in *A. oryzae*.

Keywords *Aspergillus oryzae* · Heterologous protein production · Highly efficient gene-targeting · Marker recycling · Multiple gene disruptions · Protease

Introduction

Aspergillus oryzae is an excellent host for heterologous protein production due to its high protein productivity and its safety guaranteed by its use in the manufacture of Japanese fermented foods for over a thousand years (Kitamoto 2002). In order to enhance the ability of protein production it is important to breed the host applicable to multiple rounds of genetic manipulations. Our breeding of *A. oryzae* quadruple auxotrophic host (*niaD*$^{-}$ *sC*$^{-}$ *ΔargB adeA*$^{-}$) has enabled us to manipulate as many as four genes in a single host strain (Jin et al. 2004).

In general, the production level of heterologous proteins by *A. oryzae* is much lower compared to the homologous (fungal) proteins (Tsuchiya et al. 1994; Nakajima et al. 2006; Jin et al. 2007; Ito et al. 2007). We reported that double proteinase gene disruption

Electronic supplementary material The online version of this article (doi:10.1007/s10529-008-9763-9) contains supplementary material, which is available to authorized users.

J.-I. Maruyama · K. Kitamoto (✉)
Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
e-mail: akitamo@mail.ecc.u-tokyo.ac.jp

(*tppA* and *pepE*) improved the heterologous protein production (Jin et al. 2007). The genome sequencing project revealed that *A. oryzae* has 134 protease genes (Machida et al. 2005). For multiple disruptions of protease genes, however, the number of selective markers in a single host is limited in *A. oryzae*.

The *pyrG* gene encoding orotidine-5′-phosphate (OMP) decarboxylase has been used for marker recycling, allowing multiple gene disruptions in *Aspergillus fumigatus* and *Aspergillus nidulans* (d'Enfert 1996; Nielsen et al. 2006), since positive selection for *pyrG*-excised strains can be done by using 5-fluoro-orotic acid (5-FOA) which is converted to the toxic intermediate 5-fluoro-UMP (Boeke et al. 1984). The Cre/loxP recombinase system of bacteriophage P1 also allows consecutive gene disruptions in *A. nidulans* (Forment et al. 2006). Based on these reports, however, multiple gene disruptions led to accumulation of many copies of ectopic/foreign DNA fragments in the genome. In *A. oryzae*, no attempts for marker recycling have been reported.

In order to perform multiple gene disruptions rapid and efficient methods are required. Ninomiya et al. (2004) developed a highly efficient gene-targeting system in *Neurospora crassa* by disruption of the genes encoding Ku70 and Ku80 that play a role in nonhomologous chromosomal integration. Ku-deficient strains have been employed for highly efficient gene-targeting in *A. oryzae* (Takahashi et al. 2006). Moreover, it was reported that all pathways of nonhomologous chromosomal integration in *N. crassa* are under the control of MUS-53 (human Lig4 homolog) (Ishibashi et al. 2006). The *mus-53* gene disruptant shows 100% gene-targeting frequency even with short (100-bp) flanking sequences, providing a remarkably efficient system for gene targeting. Very recently, it was shown that gene-targeting efficiency was improved by defect of the homolog of MUS-53 (LigD) in *A. oryzae* (Mizutani et al. 2008).

In this study we performed multiple gene disruptions by developing a marker-recycling system in *A. oryzae*. In order to rapidly carry out multiple gene disruptions, high targeting frequency was achieved by disruption of the gene homologous to *N. crassa mus-53*. Finally, we generated the double proteinase gene disruptant (Δ*tppA* Δ*pepE*) applicable to further multiple gene disruptions in *A. oryzae*.

Materials and methods

Strains and growth media

Aspergillus oryzae wild type strain, RIB40 (Machida et al. 2005) and quadruple auxotrophic strain, NSAR1 (Jin et al. 2004) (Supplementary Table 1) were used as a DNA donor and for transformation, respectively. The strains generated in this study are listed in Supplementary Table 1. DPY (20 g/l dextrin, 10 g/l polypeptone, 5 g/l yeast extract, 5 g/l KH_2PO_4, and 0.5 g/l $MgSO_4 \cdot 7H_2O$ (pH 5.5)) and potato/dextrose/agar (PDA) media were used for growth. M + Met medium (2 g/l NH_4Cl, 1 g/l $(NH_4)_2SO_4$, 0.5 g/l KCl, 0.5 g/l NaCl, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.02 g/l $FeSO_4 \cdot 7H_2O$, 20 g/l glucose, 1.5 g/l methionine pH 5.5) with required supplements (0.1 g/l adenine, 10–40 mM uridine, and 1–4 g/l uracil) was used for transformation and growth of *A. oryzae* strains. *Escherichia coli* DH5α (*supE44 ΔlacU169* (*Φ80 lacZ ΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for DNA manipulation.

Transformation of *A. oryzae*

Transformation of *A. oryzae* was done according to the method of Kitamoto (2002).

Molecular techniques

The recombination reactions for plasmid construction in the MultiSite Gateway system were carried out as instructed by the manufacturer (Invitrogen, San Diego, CA, USA). DNA fragments were amplified with the PrimeSTAR HS DNA Polymerase (TaKaRa, Otsu, Japan) and their nucleotide sequences were confirmed with ABI PRISM 310NT Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All primers used in this study are listed in Supplementary Table 2. Gene disruption methods in *A. oryzae* are described in Supplementary Methods.

Excision of the *pryG* marker

Conidia (approx. 10^6–3×10^6) of the gene disruptants with the *pyrG* marker were spread on PD agar medium containing 1 mg 5-FOA/ml and 20 mM uridine, and then incubated at 30°C. After 5–8-day cultivation, colonies were observed to grow and

transferred onto another 5-FOA agar medium supplemented with uridine. The 5-FOA resistant strains were examined for uridine/uracil auxotrophy. In order to verify the *pyrG* excision PCR analysis was done using the primers (tpp-1340_F and tpp + 237_R for *tppA*, pEp-488_F and pEt + 162_R for *pepE*) and genomic DNAs of transformants as template.

Southern analysis

The strains were identified by Southern analysis. After electrophoresis, the digested genomic DNAs were transferred onto Hybond N+ membrane (GE Healthcare, Piscataway, NJ, USA). ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system (GE Healthcare) and LAS-100plus luminescent image analyzer (Fuji Photo Film, Tokyo, Japan) were used for detection.

Results

Disruption of *A. oryzae ligD* gene homologous to *N. crassa mus-53* gene

We searched the gene homologous to *N. crassa mus-53* gene in *A. oryzae* Genome database DOGAN (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao). The gene with the gene ID AO090120000322 (designated as *ligD* (Mizutani et al. 2008)) was predicted to encode the 1,006 amino acid protein sharing 49.4% homology with MUS-53. For disruption of the *ligD* gene the flanking regions of the *ligD* ORF were cloned and connected with the selective marker, *argB*. The DNA fragment for disruption of the *ligD* gene was introduced into the *A. oryzae* quadruple auxotrophic strain, NSAR1 (Jin et al. 2004; Supplementary Table 1). PCR analysis using the genomic DNAs revealed that in 6 of the 16 transformants the *ligD* gene was disrupted, which showed 38% disruption efficiency (Table 1). Southern analysis was also performed to verify disruption of the *ligD* gene (Supplementary Fig. 1).

The *ligD* disruptants grew and conidiated comparably to the non-disrupted transformants (Fig. 1a). This suggests that the strains can be normally used for further experiments such as protein production. On the other hand, the Δ*ligD* strain reduced the growth in the presence of methyl methanesulfonate (MMS), a chemical mutagen (Fig. 1a). These phenotypes of the Δ*ligD* strain generated in our study were consistent with the other recent report of the *ligD* gene disruption in *A. oryzae* (Mizutani et al. 2008).

Table 1 Efficiency of gene disruption in *A. oryzae*

Disrupted gene	Selective marker	*ligD* gene in the recipient strain	Disruption/ Transformants (ratio)
ligD	*argB*	wild type	6/16 (38%)
pyrG	*adeA*	Δ	12/13 (92%)
tppA	*pyrG*	Δ	8/9 (89%)
pepE	*pyrG*	Δ	9/10 (90%)

Disruption of the *pyrG* gene was verified by uridine/uracil auxotrophy

Disruption of the *ligD*, *tppA*, and *pepE* genes were verified by PCR

Isolation of the *pyrG* disruptant and its resistance to 5-FOA

For adding uridine/uracil auxotrophy in the *ligD* disruptant, the *pyrG* gene was disrupted. The 2 kb-flanking regions of the *pyrG* ORF were cloned and connected with the selective marker, *adeA*. The disruption fragment for the *pyrG* gene was introduced into the *ligD* disruptant (NSR-ΔlD2). Transformation frequency was not significantly influenced by disruption of the *ligD* gene. Transformants were obtained on the selective medium that contained uridine for growth of uridine/uracil auxotrophic strains. Out of the 13 transformants examined, 12 showed uridine/uracil auxotrophy, which showed the high efficiency (92%) for disruption of the *pyrG* gene (Table 1). Disruption of the *pyrG* gene was verified by Southern (Supplementary Fig. 2) and PCR analyses (data not shown).

The *pyrG* disruptant (NSPlD1) could be normally transformed with the plasmid harboring the wild type *pyrG* gene. The transformants were able to grow in the absence of uridine/uracil while the *pyrG* disruptants did not form colonies on the same medium (Fig. 1b). However, only the *pyrG* disruptant showed resistance against 5-FOA (Fig. 1b). These results suggested that positive selection using 5-FOA for uridine/uracil auxotrophs could be applied to marker recycling of the *pyrG* marker in *A. oryzae*.

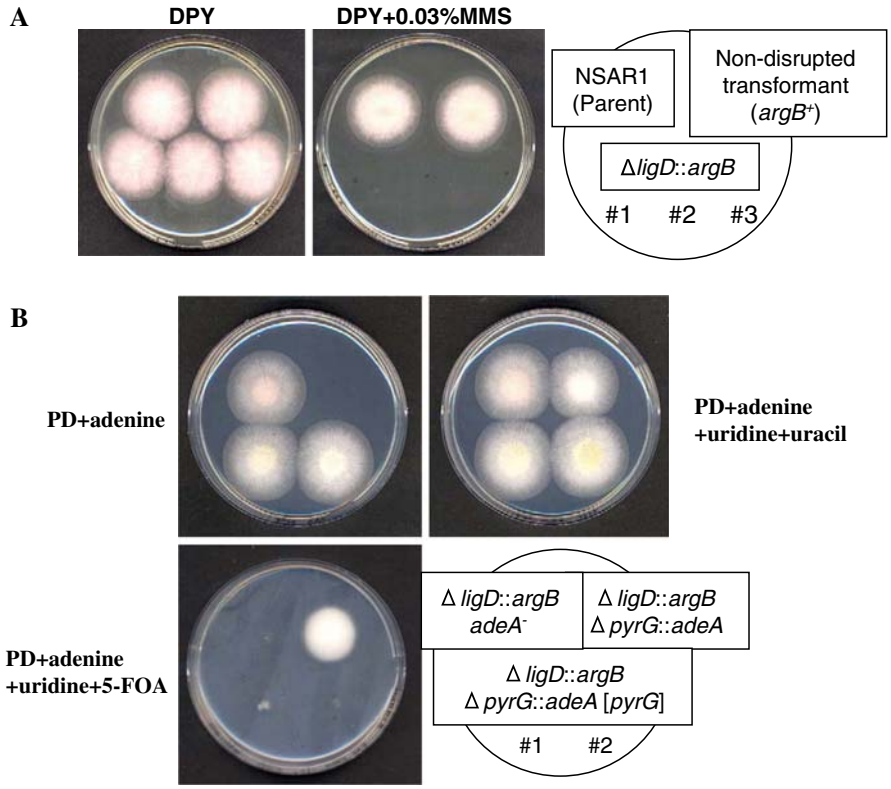

Fig. 1 Generation of the Δ*ligD*Δ*pyrG* strain in *A. oryzae*. (**a**) Growth of the *ligD* disruptant. Conidia (approximately 100 conidia/5 μl) were inoculated on the agar media and incubated at 30°C for 3 and 5 days in the absence or presence of MMS, respectively. (**b**) Growth of the Δ*ligD*Δ*pyrG* strain. Conidia (approximately 600 conidia/5 μl) were spotted on PD media with indicated supplements and 5-FOA (800 μg/ml). The agar plates were incubated at 30°C for 3 days. Δ*ligD*::*argB adeA*$^{-}$: the parental strain (NSR-ΔlD2), Δ*ligD*::*argB* Δ*pyrG*::*adeA*: the *pyrG* disruptant (NSPlD1), Δ*ligD*::*argB* Δ*pyrG*::*adeA* [*pyrG*]: the *pyrG*-complemented strains (Host: NSPlD1, Plasmid: pgEpG (See Supplementary Methods))

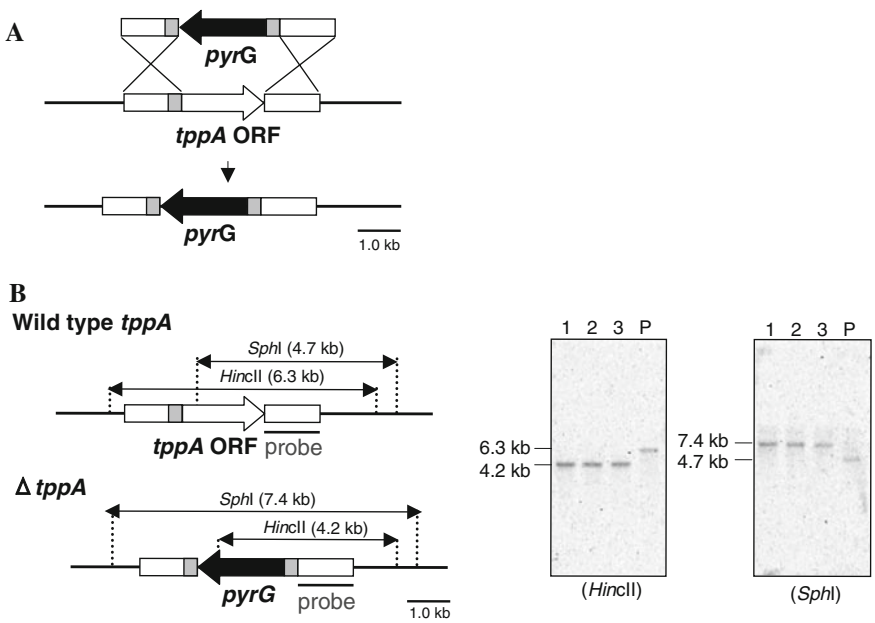

Fig. 2 Disruption of the *tppA* gene in the Δ*ligD*Δ*pyrG* background. (**a**) Schematic model of disruption of the *tppA* gene. The boxes (1.3 kb) are the flanking regions used for disruption of the *tppA* gene. The 0.3 kb upstream flanking region of the *tppA* gene (boxed in gray) was attached at 5′-end of the downstream flanking regions, introducing direct repeats. (**b**) Southern analysis of the *tppA* disruptants The genomic DNAs were digested with *Hin*cII and *Sph*I. All 3 strains analyzed in the panel (lanes 1–3) exhibited the expected band pattern for disruption of the *tppA* gene. "P" represents the parental strain (NSPlD1)

Disruption of the *tppA* gene with the *pyrG* marker

The 1.3 kb flanking regions of the *tppA* ORF were cloned and connected with the *pyrG* gene. In this construct 3′-end of the upstream flanking region of the *tppA* ORF (~300 bp) was fused with the downstream flanking region of the *tppA* ORF so that the *pyrG* marker was flanked by the ~300 bp directed repeats (Fig. 2a; gray box). The disruption fragment for the *tppA* gene was introduced into the Δ*ligD*Δ*pyrG* strain (NSPlD1). PCR analysis revealed that out of the 9 transformants, 8 showed disruption of the *tppA* gene, indicating high disruption efficiency (89%) (Table 1). Disruption of the *tppA* gene was also verified by Southern analysis (Fig. 2b).

Excision of the *pyrG* marker by using 5-FOA

Since the uridine/uracil auxotrophs were resistant to 5-FOA in *A. oryzae*, positive selection for *pyrG*$^{-}$ strains was carried out using 5-FOA. It was expected that the *pyrG* inserted at the *tppA* locus would be excised out by homologous recombination with the direct repeats, in which the flanking regions of the *tppA* ORF were directly connected without leaving any ectopic/foreign DNA fragments (Fig. 3a). Conidia of the Δ*tppA*::*pryG* strains (NSlD-tA1 ~ 3) were spread onto the agar medium containing 5-FOA and uridine. One colony per approx. 10^6 conidia appeared after 5–8-day cultivation and the resulting 5-FOA resistant strains exhibited uridine/uracil auxotrophy (Fig. 3b). The strains were confirmed for *pyrG* marker excision by PCR (data not shown) and Southern analyses (Fig. 3c). These data indicate that the *pyrG* inserted at the *tppA* locus was successfully excised by homologous recombination with the flanking direct repeats.

Successive round of gene disruption (*pepE*) and marker recycling

Next, the 1.3 kb flanking regions of the *pepE* ORF were cloned and connected with the *pyrG* gene. In this construct 3′-end of the upstream flanking region

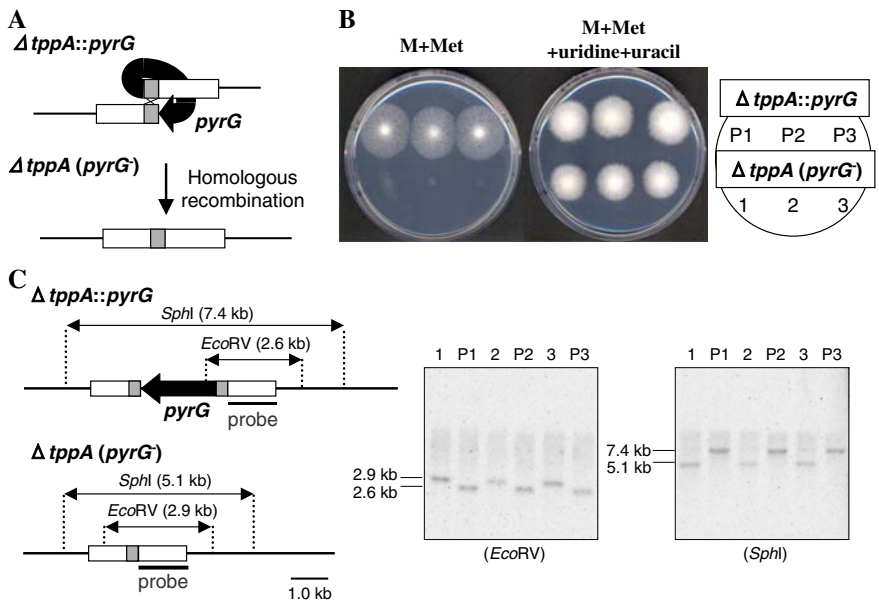

Fig. 3 Excision of the *pyrG* gene targeted at the *tppA* locus. (**a**) Schematic model of disruption of the *tppA* gene. By homologous recombination of the direct repeats consisting of the 0.3 kb upstream flanking region of the *tppA* gene (boxed in gray), the *pyrG* gene targeted at the *tppA* locus is excised, and then the upstream and downstream flanking region of the *tppA* ORF are directly connected. Note that no ectopic/foreign DNA fragments are left in the genome after excision of the *pyrG* marker. (**b**) Growth of the *pyrG*-excised strains. The conidia (approximately 500 conidia/5 μl) of the strains were spotted onto the M+Met medium with or without uridine and uracil, and then incubated at 30°C for 3 days. "P1–P3" represent the parental strains (NSlD-tA1 ~ 3). "1–3" are the *pyrG*-excised strains derived from the NSlD-tA1 ~ 3 strains. (**c**) Southern analysis of the *pryG*-excised strains. The genomic DNAs were digested with *Eco*RV and *Sph*I

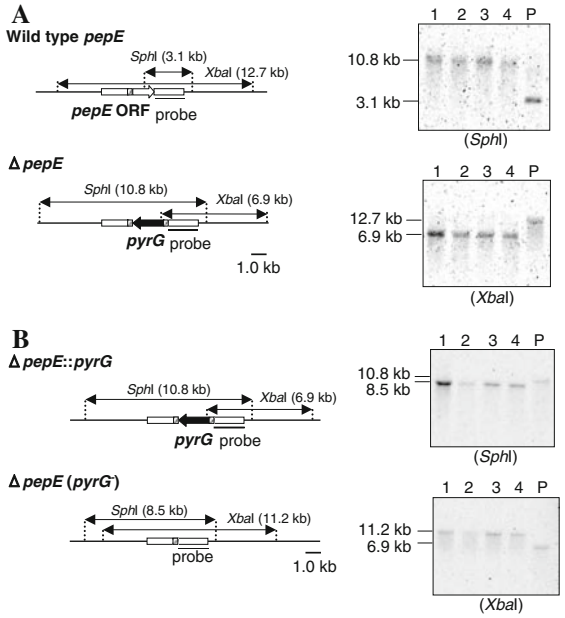

Fig. 4 Successive round of gene disruption (*pepE*) and marker recycling. (**a**) Southern analysis of the *pepE* disruptants. The boxes (2.0 and 1.9 kb) are the flanking regions for disruption of the *pepE* gene. The 0.3 kb upstream flanking region of the *pepE* ORF (hatched box) was attached at 5′-end of the downstream flanking regions, introducing direct repeats. The genomic DNAs were digested with *Sph*I and *Xba*I. All 4 strains analyzed in the panels (lanes 1–4) exhibited the expected band pattern for disruption of the *pepE* gene. "P" represents the parental strain (NSPlD-tA3). (**b**) Southern analysis of the *pyrG*-excised strains. The genomic DNAs were digested with *Sph*I and *Xba*I. All 4 strains analyzed in the panels (lanes 1–4) exhibited the expected band pattern for excision of the *pyrG* gene from the *pepE* locus. "P" represents the parental strain (NSlD-tApE2)

of the *pepE* ORF (∼300 bp) was fused with the downstream flanking region of the *pepE* ORF so that the *pyrG* marker was flanked by the ∼300 bp directed repeats (Fig. 4a; hatched box). The disruption fragment for the *pepE* gene was introduced into the Δ*ligD*Δ*pyrG*Δ*tppA* strain (NSPlD-tA3). PCR analysis revealed that out of the 10 transformants 9 showed uridine/uracil auxotrophy, which was 90% efficiency for disruption of the *tppA* gene (Table 1). Disruption of the *pepE* gene was verified by Southern (Fig. 4a) and PCR analyses (data not shown).

For another round of marker recycling, *pyrG*$^{-}$ strains were positively selected from the Δ*pepE*::*pyrG* strain (NSlD-tApE2) using 5-FOA. One colony per approximately 2.3×10^5 conidia appeared after 5–8-day cultivation on the agar medium containing 5-FOA and uridine. The 5-FOA resistant strains showed uridine/uracil auxotrophy and confirmed for *pyrG* marker excision by PCR (data not shown) and Southern analyses (Fig. 4b). Finally, we generated the double disruptant of proteinase genes (*tppA* and *pepE*), which is still applicable to successive rounds of gene disruptions.

Discussion

In order to perform multiple gene disruptions for breeding of excellent host strains, many target genes should be disrupted rapidly and efficiently. Therefore, we generated the *ligD* disruptant for highly efficient gene-disruption frequency in *A. oryzae*. The genes (*pyrG*, *tppA*, and *pepE*) could be disrupted at very high frequency (∼90%) in the Δ*ligD* background (Table 1). The disruption rate in the Δ*ligD* background is higher than the *A. oryzae* Ku70-deficient strain (Takahashi et al. 2006). This may be consistent with that *N. crassa mus-53* disruptants shows higher gene-targeting rate than the Ku-deficient strain (*mus-52* disruptant) (Ishibashi et al. 2006). Moreover, the Δ*ligD* strain grew normally and conidiated comparably to the non-disrupted transformants (Fig. 1a). These data propose that use of the Δ*ligD* strain is effective in multiple gene disruptions. However, the gene disruption efficiency could not always reach 100% in the Δ*ligD* background of *A. oryzae*. In other recent paper of the *ligD* disruptant in *A. oryzae*, some of the genes could not be disrupted at 100% efficiency (Mizutani et al. 2008). In this study we used the 1.3–2.0 kb flanking regions of target genes for disruption, while the same length of the flanking fragments led to 100% disruption frequency in *N. crassa mus-53* disruptant (Ishibashi et al. 2006). This suggests some distinct chromosomal recombination mechanism in *A. oryzae* from *N. crassa*.

This paper first describes that the multiple gene disruptions with maker recycling were done in the highly efficient gene-targeting background in filamentous fungi. We took advantage of the direct repeats consisting of the short upstream flanking region of the disrupted ORF, allowing multiple gene disruptions without leaving any ectopic/foreign DNA remnants in the genome during marker recycling. Recently, we reported that by monitoring global expression of protease genes in *A. oryzae* the *nptB* gene encoding a neutral protease was chosen from the

protease genes induced during the cultivation and that its disruption improved the production level of the heterologous protein (Kimura et al. 2008). We are planning to breed additional gene disruptant such as the *nptB* gene using the double proteinase disruptant. It is expected that our technique of multiple gene disruptions would contribute to not only further improvement of heterologous protein production but also functional analyses of many genes of interest in *A. oryzae*.

Acknowledgements We thank to Yukiko Oshima for experimental help. This study was supported by a Grant-in-Aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Boeke JD, LaCroute F, Fink GR (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet 197:345–346

d'Enfert C (1996) Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5′-decarboxylase gene, *pyrG*, as a unique transformation marker. Curr Genet 30:76–82

Forment JV, Ramón D, MacCabe AP (2006) Consecutive gene deletions in *Aspergillus nidulans*: application of the Cre/loxP system. Curr Genet 50:217–224

Ishibashi K, Suzuki K, Ando Y, Takakura C, Inoue H (2006) Nonhomologous chromosomal integration of foreign DNA is completely dependent on MUS-53 (human Lig4 homolog) in *Neurospora*. Proc Natl Acad Sci USA 103:14871–14876

Ito K, Asakura T, Morita Y, Nakajima K, Koizumi A, Shimizu-Ibuka A, Masuda K, Ishiguro M, Terada T, Maruyama J, Kitamoto K, Misaka T, Abe K (2007) Microbial production of sensory-active miraculin. Biochem Biophys Res Commun 360:407–411

Jin FJ, Maruyama J, Juvvadi PR, Arioka M, Kitamoto K (2004) Development of a novel quadruple auxotrophic host transformation system by *argB* gene disruption using *adeA* gene and exploiting adenine auxotrophy in *Aspergillus oryzae*. FEMS Microbiol Lett 239:79–85

Jin FJ, Watanabe T, Juvvadi PR, Maruyama J, Arioka M, Kitamoto K (2007) Double disruption of the proteinase genes, *tppA* and *pepE*, increases the production level of human lysozyme by *Aspergillus oryzae*. Appl Microbiol Biotechnol 76:1059–1068

Kimura S, Maruyama J, Takeuchi M, Kitamoto K (2008) Monitoring global gene expression of proteases and improvement of human lysozyme production in *nptB* gene disruptant of *Aspergillus oryzae*. Biosci Biotechnol Biochem 72:499–505

Kitamoto K (2002) Molecular biology of the Koji molds. Adv Appl Microbiol 51:129–153

Machida M, Asai K, Sano M et al (2005) Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438:1157–1161

Mizutani O, Kudo Y, Saito A, Matsuura T, Inoue H, Abe K, Gomi K (2008) A defect of LigD (human Lig4 homolog) for nonhomologous end joining significantly improves efficiency of gene-targeting in *Aspergillus oryzae*. Fungal Genet Biol (in press)

Nakajima K, Asakura T, Maruyama J, Morita Y, Oike H, Shimizu-Ibuka A, Misaka T, Sorimachi H, Arai S, Kitamoto K, Abe K (2006) Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by *Aspergillus oryzae*. Appl Environ Microbiol 72:3716–3723

Nielsen ML, Albertsen L, Lettier G, Nielsen JB, Mortensen UH (2006) Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. Fungal Genet Biol 43:54–64

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci USA 101:12248–12253

Takahashi T, Masuda T, Koyama Y (2006) Enhanced gene targeting frequency in *ku70* and *ku80* disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*. Mol Genet Genomics 275:460–470

Tsuchiya K, Nagashima T, Yamamoto Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1994) High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. Biosci Biotechnol Biochem 58:895–899