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Highly efficient gene targeting in *Aspergillus oryzae* industrial strains under *ligD* mutation introduced by genome editing: Strain-specific differences in the effects of deleting EcdR, the negative regulator of sclerotia formation

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Numerous strains of *Aspergillus oryzae* are industrially used for Japanese traditional fermentation and for the production of enzymes and heterologous proteins. In *A. oryzae*, deletion of the *ku70* or *ligD* genes involved in non-homologous end joining (NHEJ) has allowed high gene targeting efficiency. However, this strategy has been mainly applied under the genetic background of the *A. oryzae* wild strain RIB40, and it would be laborious to delete the NHEJ genes in many *A. oryzae* industrial strains, probably due to their low gene targeting efficiency. In the present study, we generated *ligD* mutants from the *A. oryzae* industrial strains by employing the CRISPR/Cas9 system, which we previously developed as a genome editing method. Uridine/uracil auxotrophic strains were generated by deletion of the *pyrG* gene, which was subsequently used as a selective marker. We examined the gene targeting efficiency with the *ecdR* gene, of which deletion was reported to induce sclerotia formation under the genetic background of the strain RIB40. As expected, the deletion efficiencies were high, around 60~80%, in the *ligD* mutants of industrial strains. Intriguingly, the effects of the *ecdR* deletion on sclerotia formation varied depending on the strains, and we found sclerotia-like structures under the background of the industrial strains, which have never been reported to form sclerotia. The present study demonstrates that introducing *ligD* mutation by genome editing is an effective method allowing high gene targeting efficiency in *A. oryzae* industrial strains.

Key Words: *Aspergillus oryzae*; genome editing; gene targeting; industrial strain; sclerotia formation

Introduction

Industrially, numerous *Aspergillus oryzae* strains are selectively used for different purposes, such as Japanese traditional fermentation and production of enzymes and heterologous proteins. In *A. oryzae*, genetic engineering based on homologous recombination has been established following the development of a transformation method and several selective markers. In addition, gene targeting efficiency in *A. oryzae* was greatly improved by generating strains by the deletion of genes (*ku70* and *ligD*) involved in non-homologous end joining (NHEJ) (Maruyama and Kitamoto, 2008; Mizutani et al., 2008; Takahashi et al., 2006), as has been similarly reported in other *Aspergillus* species (Krappmann et al., 2006; Meyer et al., 2007; Nayak et al., 2006). This strategy for genetic engineering has been applied almost exclusively under the background of the *A. oryzae* wild strain RIB40, which was used for genome sequencing (Machida et al., 2005). However, deletion of the NHEJ genes by homologous recombination would be laborious in numerous *A. oryzae* industrial strains because

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Table 1. Primers used in this study.

Primer name	Sequence 5′ to 3′
niaD-del-upF	TTGTGACCTTTCTCCGAGGCATTATATGGT
niaD-del-upR	GCACCAGCTCTTCTTTGCCATGAAGCCGTCGAGCTCAGCC
niaD-del-downF	GACGGCTTCATGGCAAAGAAGAGCTGGTGCGGTGGAGCTTGTTTT
niaD-del-downR	TGCTTCTATGGCGTCAGTCTTTAGAGCAGT
cp-niaDdel-F	CGGTCTTGGACAAAGGCAACATTGAAAGAA
cp-niaDdel-R	GTGATTATTGCTTTCATGTCGGCATTGGCT
pUNA+U6-F	ATAGGAAAGGATCCTTAATGCCGGCTCATTCAAACGGAAATACGAGGGAC
U6p-ligD-R1	ACTTCCGTGCGCATTGGCCGCACTTGTTCTTCTTTACAATGATT
ligD-U6t-F1	GCGGCCAATGCGCACGGAAGTGTTTTAGAGCTAGAAATAGCAAG
pUNA+U6p+gRNA+U6t-R3	GCAGGTCGACTCTAGAGCAGCTCTATATCACGTGACGTATTTAGA
pUC119pGup1530_F	TCGAGCTCGGTACCCGTGCGGCTGACAACTATGAT
pGdown1up20_R	GAGTACGTATCCACCACTACCGCGGGGTTCTGCAAATATT
pGdup20own1_F	AATATTTGCAGAACCCCGCGGTAGTGGTGGATACGTACTC
pUC119pGdown1457_R	CTCTAGAGGATCCCCCGCCGAGCCACTTAGTATAT
pyrGdown1522_R	GGACGGTTACAAAATCCACG
pGdn410_Fw	GGGTTGGGCTTATTGCTATG
pUC119-eRup354_F	TCGAGCTCGGTACCCTTATCAACTGTCTTTCCCGCCC
feRup1-eRdown6_R	AGAAGCAACATGCCCTGCCATTCTGTTGCGTTAGGAGCGTTG
feRdown6-eRup1_F	CAACGCTCCTAACGCAACAGAATGGCAGGGCATGTTGCTTCT
pUC119-eRdown1464_R	CTCTAGAGGATCCCCAAATGTAGGGTCGCTGGCAC
pUC119-eRup1467_F	TCGAGCTCGGTACCCCCTTCTATGGCCCGTGTAGA
feRup-pG_Rv	CAAGCCTGCTTTTTTGTACAAATTCTGTTGCGTTAGGAGCGTTG
fpG-eRup1_Fw	ACGCTCCTAACGCAACAGAATTTGTACAAAAAAGCAGGCTTGGC
feRdown6-pG_Rv	GGCGGGAAAGACAGTTGATAATTTGTACAAGAAAGCTGGGTGGT
ecdRup1751-F	TCAGTTTGGGATGCTGGACT

of their low gene targeting efficiency. Therefore, it is important to develop an effective method for inactivating the NHEJ genes for efficient gene targeting in the industrial strains.

Genome editing technologies using bacterial nucleases have been developed, facilitating genetic engineering in a wide variety of organisms (Ul Ain et al., 2015). One of these technologies, the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system is regarded as the most convenient genome editing method (Doudna and Charpentier, 2014). In this system, the Cas9 nuclease of *Streptococcus* species interacts with a single guide RNA, and is recruited to the target locus. When the DNA strand cleaved by Cas9 is repaired, mutation is introduced at the target locus. The functionality of the CRISPR/Cas9 system as a genome editing method has been reported in filamentous fungi (Arazoe et al., 2015; Fuller et al., 2015; Katayama et al., 2016; Liu et al., 2015; Nødvig et al., 2015). We recently developed the CRISPR/Cas9 system as a genome editing method in *A. oryzae* and demonstrated that the system can be applied to industrial strains (Katayama et al., 2016). However, the efficiency of mutagenesis using the CRISPR/Cas9 system was 10~20% (Katayama et al., 2016), which is lower than the gene targeting efficiencies in the deletion of *ku70* and *ligD* (Maruyama and Kitamoto, 2008; Mizutani et al., 2008; Takahashi et al., 2006). Therefore, mutation of *ku70* or *ligD* genes using the CRISPR/Cas9 system would be effective for efficient gene targeting.

One of typical characteristics of *A. oryzae* industrial strains is the disability to form a sclerotium (Murakami, 1971), which is a hardened compact mycelial mass formed for the survival in adverse environmental conditions (Willetts and Bullock, 1992). It was reported that sclerotia serve as repositories for sexual reproductive structures in *Aspergillus flavus* (Horn et al., 2009), which has been supposed to be the ancestor of *A. oryzae* (Gibbons et al., 2012). As a sexual life cycle of *A. oryzae* has not been discovered (Wada et al., 2012), sclerotia formation should be important to induce sexual reproduction using *A. oryzae* industrial strains, which would contribute to efficient breeding. It was previously reported that deletion of the *ecdR* gene, which encodes for a basic helix-loop-helix transcription factor, enhances sclerotia formation under the genetic background of the wild strain RIB40 (Jin et al., 2011). However, it remains unknown whether deletion of *ecdR* leads to sclerotia formation in the industrial strains.

In this study, we generated *ligD* mutants from *A. oryzae* industrial strains using the CRISPR/Cas9 system. Moreover, with high gene targeting efficiency in the *ligD* mutants generated, we performed *ecdR* gene deletion, indicating the possibility of sclerotia formation in the industrial strains.

Materials and Methods

***A. oryzae* strains, growth conditions and transformation.** The *A. oryzae* wild strain RIB40 (Machida et al., 2005) and industrial strains RIB128, RIB915 and RIBOIS01 (Murakami, 1971; http://www.nrib.go.jp/data/asp/strain.html) were used in this study. Potato dextrose (PD) medium (Nissui, Tokyo, Japan), Malt Extract (ME) medium (2% malt extract, 2% glucose, 0.1% polypeptone, pH 6.0), DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, pH 5.5), and Czapek-Dox (CD) medium (0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.002% $FeSO_4{\cdot}7H_2O$, and 2% glucose, pH 5.5) were used for the growth of strains. Transformation of *A. oryzae* strains was performed according to the method described previously

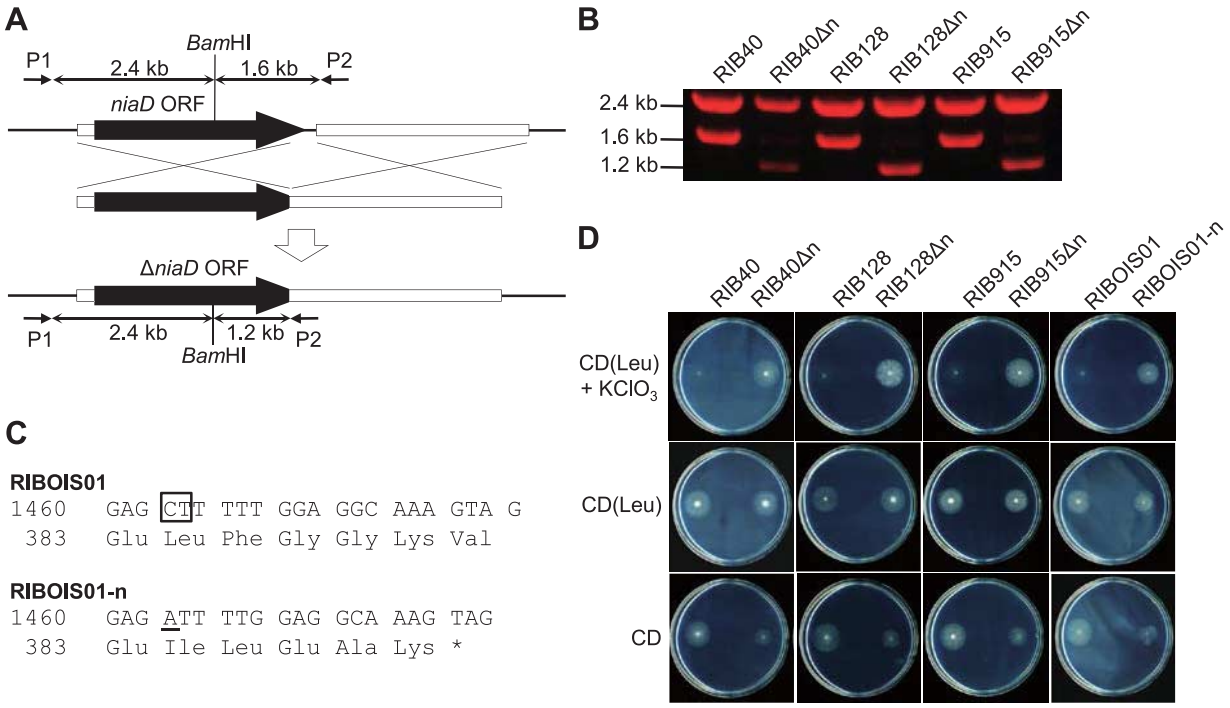

Fig. 1. Generation of the *niaD* mutants in the *A. oryzae* industrial strains.

A. The scheme for deletion of the 3′-end of *niaD*. P1 and P2 indicate the position of primers for genome PCR, cp-niaDdel-F and cp-niaDdel-R, respectively. B. Genome PCR analysis of the *niaD* mutants from the strains RIB40, RIB128 and RIB915. The amplified fragments were digested by *Bam*HI. C. Mutation within the *niaD* gene in the strain RIBOIS01-n. The box and underlining indicate deleted nucleotides and inserted nucleotide, respectively. D. Phenotype of the *niaD* mutants. Conidial suspensions (5×10^4/5 μL) were spotted on the indicated media and incubated at 30°C for 3 days.

(Maruyama and Kitamoto, 2011). CD medium was used for the selection of strains transformed with the *niaD* and *pyrG* selective markers. CD(Leu) medium, a modified CD medium containing 10 mM leucine instead of $NaNO_3$, was used for the growth of *niaD* mutants, and CD(Leu)+$KClO_3$ medium containing 470 mM $KClO_3$ (Ishi et al., 2005) was used for the selection of *niaD* mutants. CD+5-FOA+Uri/Ura medium containing 1 mg/mL 5-fluoroorotic acid (5-FOA), 0.5% uridine and 0.2% uracil was used for the selection of *pyrG* deletion mutants. PD media containing 0.5% uridine and 0.2% uracil with/without 1 mg/mL 5-FOA (referred as to PD+5-FOA+Uri/Ura and PD+Uri/Ura, respectively) were used for the growth of *pyrG* mutants. Transformants were obtained on the selective media containing 1.2 M sorbitol as the osmotic stabilizer for protoplasts, and then subcultured twice on the selective media without sorbitol.

DNA manipulation. *Escherichia coli* DH5 was used for DNA manipulation. Oligonucleotides used in this study are listed in Table 1. The polymerase chain reaction (PCR) was performed using PrimeSTAR HS DNA polymerase (TaKaRa, Otsu, Japan). The In-Fusion® HD Cloning Kit (Clontech, Palo Alto, CA, USA) was used for the plasmid construction. Nucleotide sequencing analysis of target genes was performed commercially by Fasmac Co., Ltd. (Kanagawa, Japan).

Construction of DNA fragments for deletion of the niaD gene. The DNA fragment for *niaD* mutation was constructed as follows: The fragment containing a 3′-part of the *niaD* ORF and the downstream fragment of *niaD* were amplified from the genomic DNA of RIB40 using the primer sets niaD-del-upF/niaD-del-upR, and niaD-del-downF/niaD-del-downR, respectively. These two fragments were fused using the primers niaD-del-upF and niaD-del-downR, and introduced into RIB strains. Transformants were selected on the CD(Leu)+$KClO_3$ medium and analyzed for *niaD* gene deletion by genome PCR using the primers cp-niaDdel-F and cp-niaDdel-R.

Generation of niaD mutant by spontaneous mutagenesis. Conidial suspension (3×10^4/3 μL) was spotted on the CD(Leu)+$KClO_3$ medium and incubated at 30°C for 5~7 days. Strains growing on the CD(Leu)+$KClO_3$ medium were subcultured on the same medium twice, and analyzed for *niaD* gene mutation by nucleotide sequencing.

Mutagenesis of the ligD gene. The plasmid for *ligD* gene mutation by the CRISPR/Cas9 system was constructed as follows; the *U6* promoter with the *ligD* target sequence was amplified from the plasmid pUNAFNC9gwA1 (Katayama et al., 2016) using primers pUNA+U6p-F and U6p-ligD-R1. The *U6* terminator and sgRNA sequence were amplified from pUNAFNC9gwA1 using the primers ligD-U6t-F1 and pUNA+U6p+gRNA+U6t-R3. These two fragments were fused using the primers pUNA+U6p-F and pUNA+U6p+gRNA+U6t-R3, and inserted into the *Xba*I site of the plasmid pUNAFNcas9 (Katayama et al., 2016), and the obtained plasmid pUNAFNcas9lD was introduced into the *niaD* mutants. Transformants were selected on the CD medium, and mutations in the *ligD* gene were analyzed by nucleotide sequencing.

Table 2. Mutation patterns of the *ligD* gene by the CRISPR/Cas9 system.

Strain	Mutation pattern	Nucleotide sequence around the target site*
RIB40	—	CGGTGTACTCTCAAA GCGGCCAATGCGCACGGAAGT GGGTGATATGACCATCGAGGAAGT
RIB40gC9lD	1 bp deletion	CGGTGTACTCTCAAA GCGGCCAATGCGCACGGA-GT GGGTGATATGACCATCGAGGAAGT
RIB128gC9lD	1 bp deletion	CGGTGTACTCTCAAA GCGGCCAATGCGCACGGA-GT GGGTGATATGACCATCGAGGAAGT
RIB915gC9lD	23 bp deletion	CGGTGTACTCTCAAA GCGGCCA ----------TCGAGGAAGT
RIBOIS01gC9lD	1 bp deletion	CGGTGTACTCTCAAA GCGGCCAATGCGCACGGAA-T GGGTGATATGACCATCGAGGAAGT

*Hyphens indicate the deleted nucleotides. Box and underline indicate the protospacer and protospacer-adjacent motif (PAM) sequences, respectively.

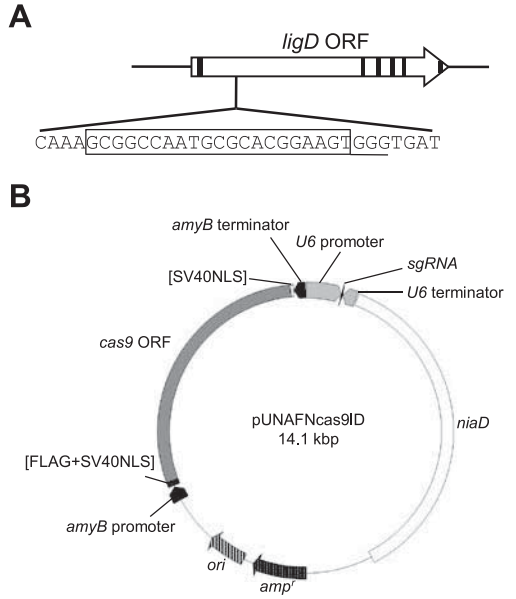

Fig. 2. Construction of the plasmid for *ligD* mutagenesis by the CRISPR/Cas9 system.

A. Position of the target sequence within the *ligD* gene. The white arrow indicates the *ligD* ORF, and the black bars within the *ligD* ORF indicate introns. The black rectangle and underbar indicate the protospacer and protospacer-adjacent motif (PAM) sequences, respectively. B. A plasmid map of pUNAFNcas9lD.

Deletion of the pyrG gene. The plasmid for *pyrG* gene deletion was constructed as follows; the 1.5 kb upstream and downstream fragments of *pyrG* were amplified from the genomic DNA of RIB40 using the primer sets pUC119pGup1530_F/pGdown1up20_R, and pGdup20own1_F/pUC119pGdown1457_R, respectively. These fragments were fused using the primers pUC119pGup1530_F and pUC119pGdown1457_R, and inserted into the *Sma*I site of pUC119 (TaKaRa, Otsu, Tokyo), yielding the plasmid pUC119ΔpG. The fragment for *pyrG* gene deletion was amplified from pUC119ΔpG using primers pUC119pGup1530_F and pUC119pGdown1457_R, and introduced into the *ligD* mutants. Transformants were selected on the CD+5-FOA+Uri/Ura medium, and analyzed for *pyrG* gene deletion by genome PCR using the primers pGdn410_Fw and pyrGdown1522_R.

Deletion of the ecdR gene. The plasmid for *ecdR* gene deletion was constructed as follows; the 0.4 kb upstream and 1.5 kb downstream fragments of *ecdR* were amplified from the genomic DNA of the strain RIB40 using the primer sets pUC119-eRup354_F/feRup1-eRdown6_R, and feRdown6-eRup1_F/pUC119-eRdown1464_R, respectively. These fragments were fused using pUC119-eRup354_F and pUC119-eRdown1464_R and inserted into the *Sma*I site of pUC119, yielding pUC119eRupdown. The fragment containing the 0.4 kb upstream and 1.5 kb downstream of *ecdR* was amplified from pUC119eRupdown using the primers feRud-pG and pUC119-eRdown1464_R. The 1.5 kb upstream fragment of *ecdR* was also amplified from the genomic DNA of RIB40 using the primers pUC119-eRup1467_F and feRup1-pG_Rv. The *pyrG* marker gene was amplified from the plasmid pgEpG (Maruyama and Kitamoto, 2008) using the primers fpG-eRup1_Fw and feRdown6-pG_Rv. These three fragments were fused using the primers pUC119-eRup1467_F and pUC119-eRdown1464_R, and inserted into the *Sma*I site of pUC119, yielding pUC119ΔeRpG. The fragment for *ecdR* deletion was amplified from pUC119ΔeRpG using the primers pUC119-eRup1467_F and pUC119-eRdown1464_R, and introduced into the *ligD* and *pyrG* double mutants. Transformants were selected on the CD medium, and analyzed by genome PCR using the primers ecdRup1751-F and pUC119eRdown1464_R.

Results and Discussion

For generating a highly-efficient gene-targeting background in the *A. oryzae* industrial strains, RIB128, RIB915 and RIBOIS01 were selected as model strains, and the wild strain RIB40 was used as control. RIB128, RIB915, RIBOIS01 are the strains used for the production of sake, soy sauce and *ginjo*-sake, respectively (Murakami, 1971; http://www.nrib.go.jp/data/asp/strain.html). In our recent report, the *niaD* gene was used as a selective marker for mutagenesis by the CRISPR/Cas9 system in *A. oryzae* strains (Katayama et al., 2016). Hence, we generated *niaD* mutants from the *A. oryzae* industrial strains. Strains RIB40, RIB128, and RIB915 were transformed with the DNA fragment for *niaD* deletion (Fig. 1A), and transformants were screened with the selective medium containing $KClO_3$, in which only *niaD* mutants can grow (Ishi et al., 2005). The deletion of *niaD* was confirmed by genome PCR, and the mutants were designated RIB40Δn, RIB128Δn, and RIB915Δn (Fig. 1B). As any *niaD* deletion strains could not be obtained from RIBOIS01, we selected spontaneous *niaD* mutants from RIBOIS01 on the $KClO_3$-containing medium (see Section "Materials and

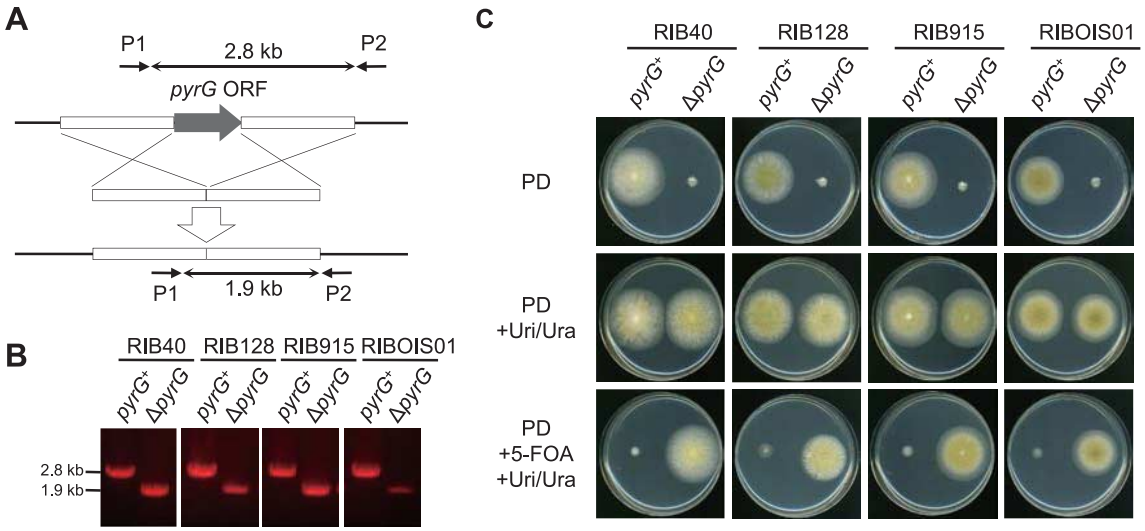

Fig. 3. Generation of the *pyrG*-deleted industrial strains.

A. The scheme for deletion of *pyrG*. P1 and P2 indicate the position of primers for the genome PCR, pGdn410_Fw and pyrGdown1522_R, respectively. B. Genome PCR analysis of the *pyrG* deletion mutants. C. Phenotypes of the *pyrG* deletion strains. Conidial suspensions (1×10^4/5 μL) were spotted on the indicated media and incubated at 30°C for 3 days.

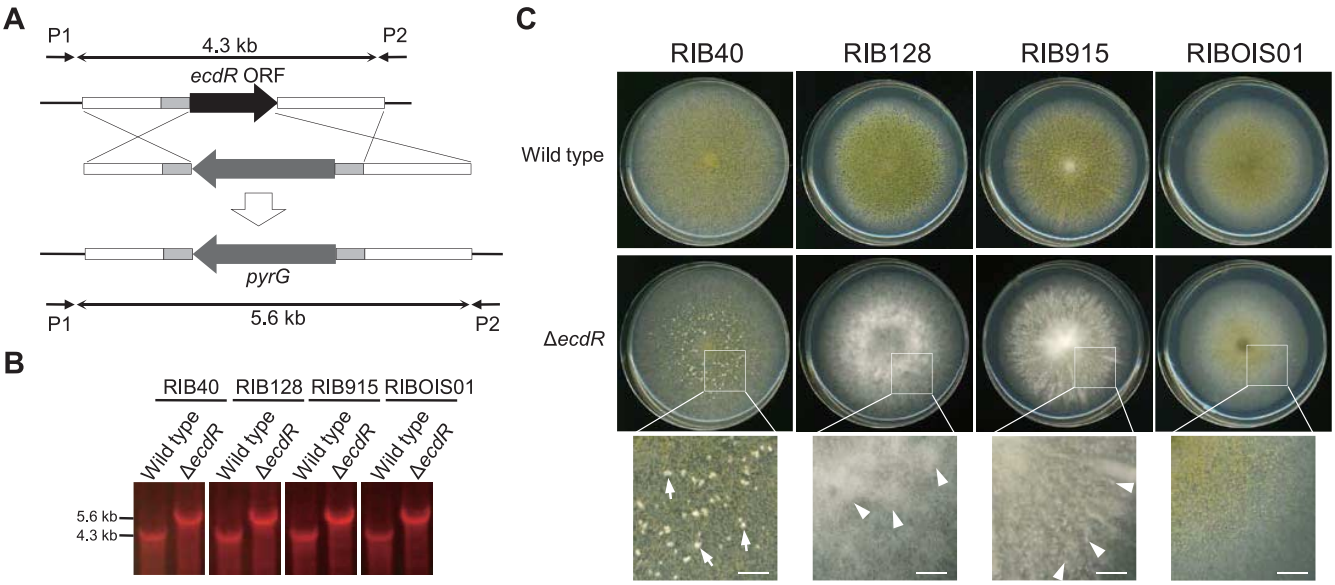

Fig. 4. Generation and characterization of the *ecdR*-deleted industrial strains.

A. The scheme for *ecdR* deletion. P1 and P2 indicate the position of the genome PCR primers, ecdRup1751-F and pUC119eRdown1464_R, respectively. B. Genome PCR analysis of the *ecdR* deletion strains. C. Phenotypes of the *ecdR*-deleted industrial strains. Conidial suspensions (1×10^4/5 μL) were spotted on the ME medium and incubated at 30°C for 5 days. Arrows and arrowheads indicate sclerotia and sclerotia-like structures, respectively. Bars: 5 mm.

Methods"). In the *niaD* mutant from RIBOIS01, which was designated RIBOIS01-n, the nucleotides 1463C and 1464T were replaced with an adenine nucleotide, which resulted in a stop codon appearing by frameshift mutation (Fig. 1C). As shown in Fig. 1D, the *niaD* mutants obtained from RIB40, RIB128, RIB915 and RIBOIS01 were able to grow on the $KClO_3$-containing medium (CD(Leu)+$KClO_3$) as well as the minimal medium with leucine used as nitrogen source (CD(Leu)), and they showed smaller growth rates than the parent strains in the presence of nitrate as nitrogen source (CD).

To construct *ligD* mutants, we designed the protospacer sequence within the *ligD* gene (Fig. 2A) and constructed the plasmid pUNAFNcas9lD (Fig. 2B). We then introduced pUNAFNcas9lD into the *niaD* mutants and selected transformants assimilating nitrate as a nitrogen source. Nucleotide sequencing revealed that transformants containing *ligD* mutations were successfully obtained (Table 2); these transformants were designated RIB40gC9lD, RIB128gC9lD, RIB915gC9lD, and RIBOIS01gC9lD. RIB40gC9lD, RIB128gC9lD, and RIBOIS01gC9lD contain 1 bp deletion at the target sequence of the *ligD* gene, while RIB915gC9lD contains 23 bp deletion (Table 2). These mutations all cause frameshifts, which suggests that the *ligD* gene does not function in the strains.

Because auxotrophic markers were not available in the

ligD mutants generated, we obtained uridine/uracil auxotrophic strains by deleting the *pyrG* gene, which could be a selective marker used in the generation of multiple gene deletions (Maruyama and Kitamoto, 2008). RIB40gC9lD, RIB128gC9lD, RIB915gC9lD, and RIBOIS01gC9lD, were transformed with the DNA fragment for *pyrG* deletion (Fig. 3A). Transformants were selected with 5-FOA, which selectively allows the growth of *pyrG* deletion strains (Maruyama and Kitamoto, 2008). Deletion of the *pyrG* gene was confirmed by genome PCR in the 5-FOA-resistant transformants, which were then designated RIB40gC9PlD, RIB128gC9PlD, RIB915gC9PlD, and RIBOIS01gC9PlD (Fig. 3B). As expected, these strains exhibited uridine/uracil auxotrophy and resistance to 5-FOA (Fig. 3C).

To investigate gene targeting efficiencies in the obtained *ligD* and *pyrG* double mutants, we sought to delete the *ecdR* gene. As it was previously reported that deletion of *ecdR* suppresses conidiation and enhances sclerotia formation under the genetic background of the wild strain RIB40 (Jin et al., 2011), it would be possible that deletion of *ecdR* leads to sclerotia formation in the *A. oryzae* industrial strains, which have never formed any sclerotia. We introduced the DNA fragment for *ecdR* deletion with the *pyrG* marker into the strains RIB40gC9PlD, RIB128gC9PlD, RIB915gC9PlD, and RIBOIS01gC9PlD (Fig. 4A). The obtained transformants with uridine/uracil prototrophy were analyzed by genome PCR for the *ecdR* gene deletion (Fig. 4B). Fifteen transformants were examined for each strain. The gene deletion efficiency in RIB40gC9PlD was 73% (11/15), of which the gene targeting efficiency was higher than that of the strain possessing the wild type *ligD* and *ku70* genes (Maruyama and Kitamoto, 2008; Mizutani et al., 2008; Takahashi et al., 2006). Moreover, the efficiencies of *ecdR* deletion in RIB128gC9PlD, RIB915gC9PlD, and RIBOIS01gC9PlD were 60% (9/15), 73% (11/15), and 80% (12/15), respectively. The *ecdR* deletion strains derived from each of the RIB strains showed similar morphological phenotypes (data not shown). These data indicate that mutagenesis of *ligD* by the CRISPR/Cas9 system was effective for highly-efficient gene targeting in the *A. oryzae* industrial strains.

The representative *ecdR* deletion strains, which was confirmed by genome PCR (Fig. 4B), were designated RIB40gC9lDeR, RIB128gC9lDeR, RIB915gC9lDeR, and RIBOIS01gC9lDeR. In the *ecdR* deletion strain derived from the wild strain RIB40, conidiation was decreased, and sclerotia formation was enhanced (Fig. 4C), as reported previously (Jin et al., 2011). In the *ecdR*-deleted industrial strains, conidiation was decreased, but sclerotia were not formed (Fig. 4C). However, larger and smaller structures consisting of hyphal masses were found in the *ecdR* deletion strains derived from RIB128 and RIB915, respectively (Fig. 4C). Since sclerotia consist of hardened masses of hyphae (Dyer and O'Gorman, 2012), the structures found here were sclerotia-like. Therefore, it was assumed that the industrial strains RIB128 and RIB915 have additional defects in the aggregation and enlargement of hyphal masses, respectively, during sclerotia formation. In contrast, no sclerotia and sclerotia-like structures were found under the background of RIBOIS01 (Fig. 4C), suggesting that the strain RIBOIS01 has defects in the early stage of sclerotia formation.

The present study enabled highly-efficient gene targeting in *A. oryzae* industrial strains by combining the genome editing and defective NHEJ. By efficiently deleting the *ecdR* gene, sclerotia-like structures were found in the *A. oryzae* industrial strains that have never been reported to form the sclerotia. Moreover, the effects of the *ecdR* gene deletion varied among the *A. oryzae* strains tested. The technology developed here could be applied not only to genetic engineering to enhance industrial utilities, but also to the genetic investigation of strain-specific properties in numerous *A. oryzae* industrial strains.

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