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ORIGINAL RESEARCH PAPER

Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*

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Abstract
Objectives To develop a genome editing method using the CRISPR/Cas9 system in *Aspergillus oryzae*, the industrial filamentous fungus used in Japanese traditional fermentation and for the production of enzymes and heterologous proteins.
Results To develop the CRISPR/Cas9 system as a genome editing technique for *A. oryzae*, we constructed plasmids expressing the gene encoding Cas9 nuclease and single guide RNAs for the mutagenesis of target genes. We introduced these into an *A. oryzae* strain and obtained transformants containing mutations within each target gene that exhibited expected phenotypes. The mutational rates ranged from 10 to 20 %, and 1 bp deletions or insertions were the most commonly induced mutations.
Conclusions We developed a functional and versatile genome editing method using the CRISPR/Cas9 system in *A. oryzae*. This technique will contribute to the use of efficient targeted mutagenesis in many *A. oryzae* industrial strains.

Keywords *Aspergillus oryzae* · CRISPR/Cas9 system · Filamentous fungi · Genome editing

Introduction

Aspergillus oryzae is used in traditional Japanese fermentation of food such as sake, soy sauce, and miso. It is also used for the production of enzymes and heterologous proteins, and many *A. oryzae* strains are used by industry. Genetic engineering based on homologous recombination has previously been performed in *A. oryzae* following the establishment of a transformation method and the development of auxotrophic markers. Moreover, the generation of strains carrying deletion of genes (*ku70* and *ligD*) involved in non-homologous end joining, enabling efficient homologous recombination, has dramatically facilitated the genetic manipulation of *A. oryzae* (Jiang et al. 2013). However, this technique has been applied almost exclusively to laboratory strains derived from the wild strain RIB40. The deletion of *ku70* or *ligD* by homologous recombination would be more laborious in *A. oryzae* industrial strains because their multinucleate conidia make it difficult to isolate homokaryotic transformants (Maruyama et al. 2001). Therefore, a

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versatile and convenient technology for genetic engineering is required for the efficient molecular breeding of industrial strains.

Recently, several genome editing technologies using bacterial nucleases have been established (Ul Ain et al. 2015). One of these, the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system, involves only two components: the Cas9 nuclease of *Streptococcus* species and a single guide RNA (sgRNA). sgRNAs bind with Cas9 and recruit it to a specific genomic locus by forming an RNA/DNA hybrid at the protospacer sequence, which is approx. 20 bp long. 5′-NGG-3′ protospacer-adjacent motifs (PAM) are required for the stable binding of Cas9 to the target locus. When DNA double-strand breaks introduced by Cas9 are repaired, mutations are introduced at the locus.

The CRISPR/Cas9 system has been applied to various organisms such as yeasts, fish, plants, and mammalian cells (Doudna and Charpentier 2014). Its use has also been reported in filamentous fungi (Liu et al. 2015; Arazoe et al. 2015; Nødvig et al. 2015; Fuller et al. 2015). In the present study, we developed the CRISPR/Cas9 system as a genome editing method for use in *A. oryzae*.

Materials and methods

Aspergillus oryzae strains and transformation

To amplify DNA fragments by PCR, we used genomic DNA from the *A. oryzae* wild strain RIB40 (Machida et al. 2005). The *A. oryzae* laboratorystrain niaD300 (*niaD*$^{-}$), derived from RIB40 (Minetoki et al. 1996), was used as the parental strain to introduce the CRISPR/Cas9 system. Transformation of *A. oryzae* was performed according to the method described previously (Maruyama and Kitamoto 2011). Obtained transformants were subcultured twice on selective medium, and nucleotide sequencing analysis of target genes was performed commercially by Fasmac Co., Ltd.

Codon optimization of *cas9* and plasmid construction

The CRISPR/Cas9 vector was constructed as follows: codon usage of *cas9* with a sequence encoding a SV40 nuclear localization signal (SV40NLS: PKKKRKV) at its 3′-end was optimized, and synthesized by GeneArt gene synthesis (Life Technologies). The optimized *cas9* was extended by two PCR steps using primers FLAG-NLS-cas9-F, IF5-FLAG-NLS-F, and CAS9-in-fusion-6R to attach a FLAG-tag and another SV40NLS at the *N*-terminal end of Cas9 (Supplementary Material). The extended *cas9* was inserted between the *amyB* promoter and terminator in pUNA containing *niaD* as a selection marker, yielding pUNAFNcas9.

The CRISPR/Cas9 plasmids containing sgRNA encoding sequences (hereafter sgRNA sequences) were constructed as follows:the *U6*promoter was amplified from the genomic DNA of the*A. oryzae* wild strain RIB40 using the forward primer pUNA+U6p-F and reverse primer attached with each protospacer sequences targeting for*wA*, *yA*, and *pyrG* (Supplementary Table 1). The *U6* terminator and sgRNA sequence without protospacer sequence were amplified by annealing two primer combinations, gRNA+U6t-F2 and gRNA+U6t-R2, and gRNA+U6t-F1 and gRNA+U6t-R1, respectively. These two fragments were fused using the reverse primer pUNA+U6t-R3 and forward primer containing each protospacer sequence (Supplementary Table 1). The *U6* promoter and terminator with the sgRNA sequence were then fused and inserted into the*Xba*I site of pUNAFNCas9 (see Fig. 1a), yielding pUNAFNC9 gwA1–4, pUNAFNC9gyA and pUNAFNC9gpG (Fig. 2a) for the targeted mutagenesis of *wA*, *yA* and *pyrG*, respectively. The sequence of sgRNA is shown in Supplementary Material.

Preparation of cell extracts and western blot analysis

This was performed as described in Supplementary Methods.

Results and discussion

Construction of CRISPR/Cas9 plasmids

We attached two SV40NLS encoding sequences of the 5′- and 3′-ends of the codon-optimized *cas9* to enhance the nuclear localization of Cas9 nuclease. The FLAG-tag encoding sequence was attached to the

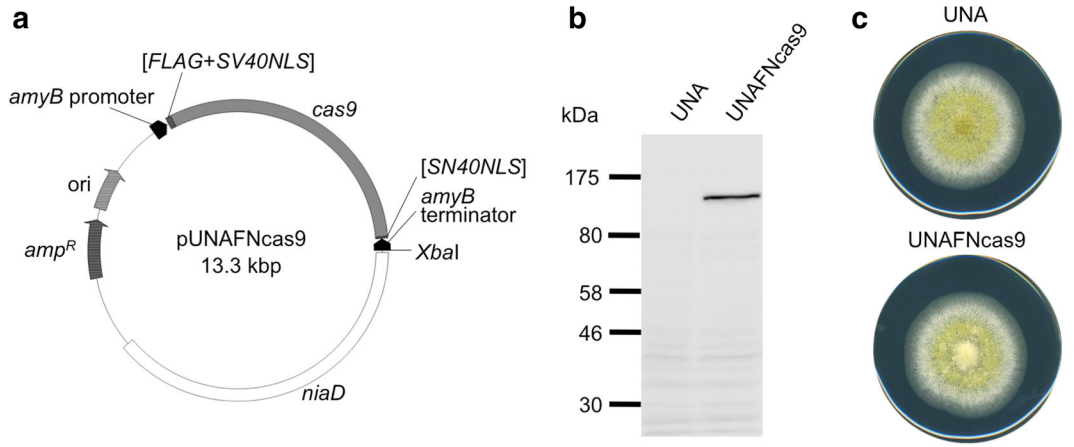

Fig. 1 Construction of a CRISPR/Cas9 vector. **a** A plasmid map of pUNAFNcas9. **b** Expression of Cas9 in strain UNAFNcas9. Western blot analysis with an anti-FLAG antibody was performed using cell extracts of strains UNA and UNAFNcas9 grown in DPY medium at 30 °C for 20 h. **c** Growth of strain UNAFNcas9. Conidial suspensions (10^4 conidia/5 µl) of strains UNA and UNAFNcas9 were inoculated onto PD medium and incubated for 4 days at 30 °C

5′-end of the codon-optimized *cas9* to confirm Cas9 expression. The resulting gene was inserted into pUNA containing the *amyB* promoter/terminator and *niaD* selection marker, yielding plasmid pUNAFNcas9 (Fig. 1a). Cas9 was detected as a single band of the predicted size (approx.160 kDa) in the transformant carrying pUNAFNcas9 (strain UNAcas9) but not in that carrying pUNA (strain UNA) (Fig. 1b). Strain UNAcas9did not exhibit any growth defect (Fig. 1c), indicating that expression of Cas9 is not detrimental to the growth of *A. oryzae*.

In the CRISPR/Cas9 system, a promoter and terminator of the *U6* small nuclear RNA (snRNA) gene are often used for sgRNA transcription to avoid the attachment of a cap structure and poly-A tail to the sgRNAs (Cong et al. 2013). We identified an ortholog of the mammalian *U6* snRNA gene, and determined its transcription initiation and termination sites on the basis of RNA-seq data in the comprehensive *A. oryzae* genome database (http://nribf21.nrib.go.jp/CFGD/). We used a 568 bp sequence upstream of the transcription initiation site and a 138 bp sequence downstream of the transcription termination site as the *U6* promoter and terminator, respectively (Supplementary Material). sgRNA sequences including protospacer sequences targeting the genes were fused with the *U6* promoter and terminator and then inserted into pUNAFNcas9 (Fig. 2a).

Mutagenesis using the CRISPR/Cas9 system

To examine the functionality of the CRISPR/Cas9 system, we carried out mutagenesis of*wA*, *pyrG*, and *yA* (AO090102000545, AO090011000868, and AO090011000755, respectively). *wA* encodes a polyketide synthase required for conidial pigmentation, and the *wA* mutant forms white conidia (Fernandez et al. 2012). *A. oryzaeyA* is an ortholog of *A. nidulans yA*, which encodes conidial laccase, and the *yA* mutant forms yellow conidia (Clutterbuck 1972). *pyrG* encodes an orotidine 5′-phosphate decarboxylase, and the deletion mutant is auxotrophic for uridine and uracil (Maruyama and Kitamoto 2011).

We selected four protospacer sequences from *wA*, wA1–4 (Supplementary Fig. 1), and introduced the CRISPR/Cas9 plasmids for *wA* mutagenesis (Fig. 2a) into *A. oryzae* strain niaD300. Nucleotide sequencing revealed that we successfully obtained transformants containing *wA* mutations in all cases using these four protospacer sequences (Table 1, Supplementary Fig. 2). We also constructed plasmids for *yA* and *pyrG* mutagenesis (Fig. 2a), and obtained mutants of these genes after transformation (Table 1). The *wA* and *yA* mutants formed white and yellow conidia, respectively (Fig. 2b), while green conidia were formed in strain UNA and the transformant carrying pUNAgwA1, which express only the *wA*-targeting sgRNA but not Cas9 (strain UNAwA1) (Fig. 2b). The

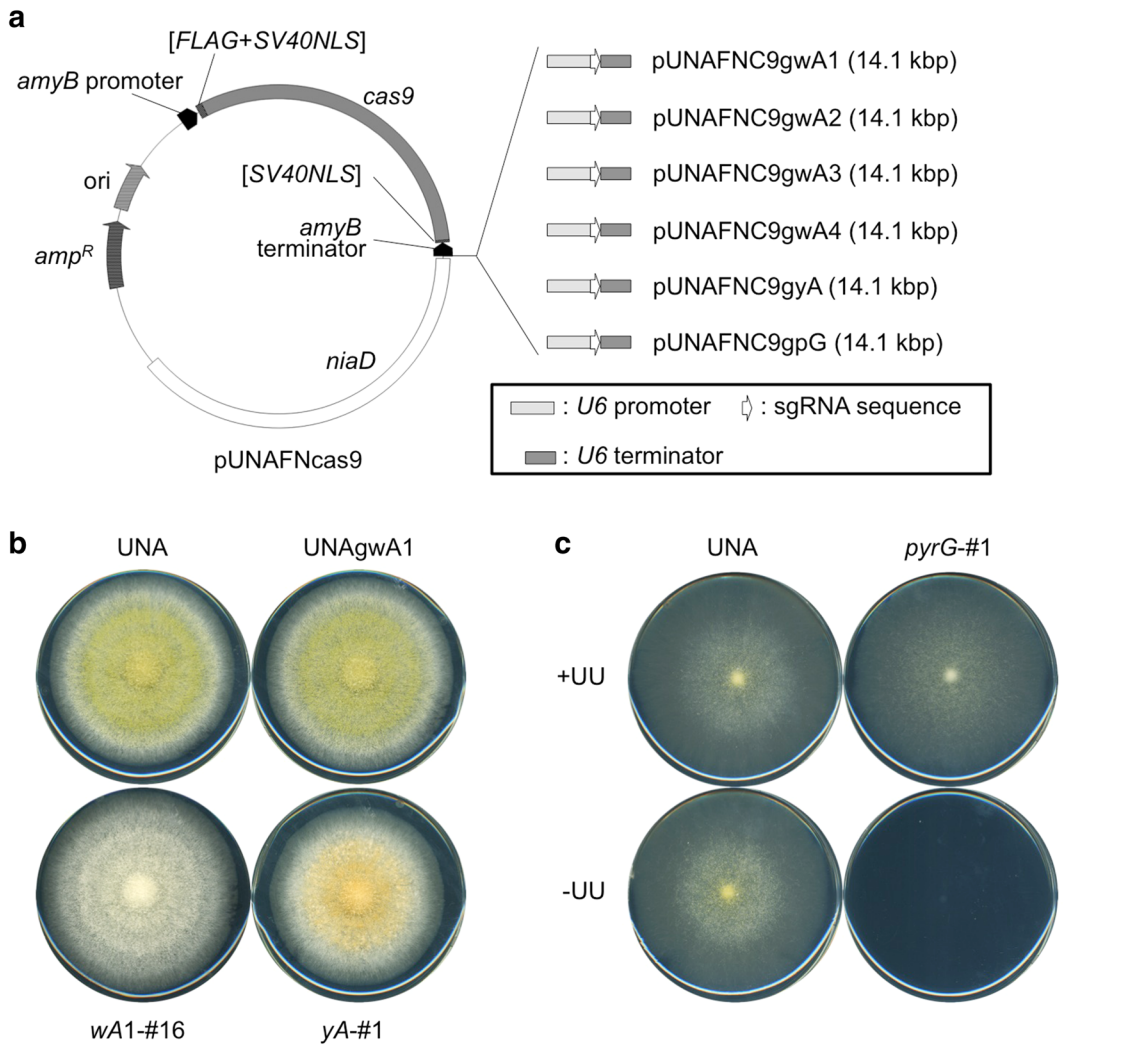

Fig. 2 Phenotypes of mutants obtained by the CRISPR/Cas9 system. **a** Construction of plasmids for the mutagenesis of *wA*, *yA*, and *pryG*. **b** Phenotypes of *wA* and *yA* mutants. Conidial suspensions (10^4 conidia/5 µl) of each strain were inoculated onto PD medium and incubated for 5 days at 30 °C. **c**Uridine and uracil auxotrophy of the *pyrG* mutant. Conidial suspensions (10^4 conidia/5 µl) of each strain were inoculated onto CD medium with uridine and uracil (+UU) and without uridine and uracil (−UU), and incubated for 6 days at 30 °C

pyrG mutant exhibited uridine and uracil auxotrophy (Fig. 2c).

When four protospacer sequences were targeted for *wA* mutagenesis, mutantional rates were 10–20 % (Table 1). *pyrG* mutants were obtained at a rate of 10 %, while that of *yA* mutants was 100 %. These results suggest that the mutational rate depends on difference of genes or protospacer sequences.

With the exception of *yA* mutagenesis, mutational rates ranged from 10 to 20 % in *A. oryzae* (Table 1). Previously reported rates using the CRISPR/CAS9 system in *Pyricularia oryzae* and *Aspergillus fumigatus* were 36–84 % and 25–53 %, respectively (Arazoe et al. 2015; Fuller et al. 2015). Conidia of *P. oryzae* and *A. fumigatus* are mononuclear, while those of *A. oryzae* are multinuclear (Maruyama et al. 2001). Because the isolation of conidia containing only mutated nuclei is difficult, the mutation efficiency of *A. oryzae* would be expected to be lower than those of *P. oryzae* and *A. fumigatus*. Indeed, genome editing using the CRISPR/Cas9 system is reportedly less efficient in *Aspergillus carbonarius* and *Aspergillus*

Table 1 Patterns of mutations introduced by the CRISPR/Cas9 system and ratios of mutants

Plasmid name	Target gene	Protospacer and PAM sequence[a]	Mutational rate (mutants/transformants)	Mutation (number of mutants)	Mutated target sequence[b]
pUNAFNC9gwA1	*wA*	GGCTGTCTAAGAGCCATAAGCCGG	18 % (7/40)	1 bp deletion (3)	GGCTGTCTAAGAGCCAT-AGCCGG
				7 bps deletion (4)	GGCTGTCTAAGAG——CCGG
pUNAFNC9gwA2	*wA*	GTGGATCTACTGGCGCGTCACCGG	10 % (2/20)	1 bp insertion (1)	GTGGATCTACTGGCGCGTA*C*ACCGG
				11 bps deletion (1)	GTGGATCTACT——GG
pUNAFNC9gwA3	*wA*	GAAAGATGCCTCGCAGCTTATAGG	20 % (2/10)	1 bp insertion (2)	GAAAGATGCCTCGCAGC*T*TTATAGG
pUNAFNC9gwA4	*wA*	GAACTAAGAAGACCCGCGCACAGG	10 % (1/10)	22 bps deletion (1)	22 bps-CAGG
pUNAFNC9gyA	*yA*	GCGCCAAATGATTCTCACTAATGG	100 % (3/3)	1 bp insertion (3)	GCGCCAAATGATTCTCACCTAATGG
pUNAFNC9gpG	*pyrG*	GACTTCCCCTACGGCTCCGAGAGG	10 % (1/10)	1 bp deletion (1)	GACTTCCCCTACGGCTC-GAGAGG

[a] Underlines indicate the PAM sequences

[b] Italic letters indicate the inserted nucleotides. Hyphens indicate deleted nucleotides

luchuensis, which contain 2–5 nuclei in each conidium (Nødvig et al. 2015).

When analyzing mutations in the present study, we frequently observed 1 bp deletions or insertions (Table 1). These were also often seen in *A. nidulans* and *Aspergillus aculeatus*, whereas large 7–83 bp deletions occurred in other *Aspergillus* species (Nødvig et al. 2015). Hence, the mutation pattern appears to vary by species. In most cases of our study, mutations were introduced 3–4 bp upstream of PAM sequences in *A. oryzae* (Table 1), which is similar to other reports using *A. nidulans* and *A. fumigatus* (Nødvig et al. 2015; Fuller et al. 2015). These findings suggest that DSB by Cas9 nuclease mainly occurs around 3–4 bp upstream of the PAM sequence in filamentous fungi.

To investigate the practicality of the CRISPR/Cas9 system in *A. oryzae* industrial strains, we attempted mutagenesis in strains RIB128 and RIB915 used for sake and soy sauce production, respectively (http://www.nrib.go.jp/data/asp/strain.html). We transformed pUNAFNC9gwA3 into *niaD*-disrupted strains derived from RIB128 and RIB915, and successfully obtained *wA* mutants (data not shown). This indicates that the genome editing method using the CRISPR/Cas9 system can be applied to *A. oryzae* industrial strains.

Conclusion

We developed the CRISPR/Cas9 system as a genome editing method for use in *A. oryzae*, and showed its functionality in both *A. oryzae* laboratory and industrial strains. This technique will enhance the convenience of genetic engineering in a wide range of *A. oryzae* industrial strains, and contribute to their efficient molecular breeding.

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Supporting information Supplementary Methods—DNA manipulation, Media, and Preparation of protein extracts and western blot analysis.

Supplementary Material—Sequences of sgRNA, *U6* promoter, *U6* terminator, and codon-optimized *cas9*.

Supplementary Table 1—Primers used in this study.

Supplementary Fig. 1—Designation of protospacer sequences for the *wA* mutagenesis.

Supplementary Fig. 2—Sequencing analyses of the target sequence within the *wA* gene in two independent *wA* mutants.

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