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CRISPR/Cas9 improves targeted knock-in efficiency in *Aspergillus oryzae*

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ABSTRACT

Aspergillus oryzae is an important fungus in food and industrial enzyme production. In *A. oryzae*, targeted knock-in transformation is primarily limited to homologous recombination (HR)-based systems, in which non-homologous end-joining (NHEJ)-disruptant hosts are required. However, preparation of hosts and transformation templates for such systems is laborious, in addition to other disadvantages. In the present study, we examined alternative targeted knock-in mediated by CRISPR/Cas9, in which a microhomology-mediated end-joining (MMEJ) and single-strand annealing (SSA) repair system was employed. This approach enabled the efficient development of targeted knock-in transformants without host preparation using only a short homology template. We conclude that this new method could be applied to facilitate the transformation of *A. oryzae*, and will make it easier to acquire targeted knock-in transformants, especially from industrially important non-model strains.

1. Introduction

Aspergillus oryzae is an important fungus species used for the production of traditional fermented foods, such as sake, shochu, shoyu, and miso.[1] It is also useful for producing heterologous proteins and secondary metabolites.[2,3] Genetic engineering using this fungus species has been extensively studied for its fundamental and practical importance. In *A. oryzae*, targeted genetic engineering by classical transformation is laborious owing to the low frequency of homologous recombination (HR). The host strain needs to be modified to improve the HR efficiency by disturbing the non-homologous end-joining (NHEJ) repair pathway via the disruption of *ligD* or *ku70*.[4,5] Recently, genome editing technologies, such as transcription activator-like effector nucleases (TALENs)[6] and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9),[7] have been successfully applied in the genetic engineering of *A. oryzae*.[8,9] These genome editing technologies have enabled the acquisition of targeted knock-out mutants without the preparation of a host strain, as mentioned above. On the other hand, acquiring targeted knock-in mutants is still laborious since it is based on HR, wherein long length homology (usually >500 bp) of the template is essential.[4,10] Recently, two alternative DNA double-strand break (DSB) repair pathways, namely microhomology-mediated end-joining (MMEJ) and single-strand annealing (SSA), have been employed as targeted knock-in methods in combination with genome editing technologies in mammalian cells.[11] Compared with HR, MMEJ/SSA requires a shorter homology length of the template, thereby making template preparation easier. However, the application of MMEJ/SSA in *A. oryzae* has not been sufficiently studied. Therefore, in the present study, we examined the efficiency of targeted knock-in mediated by CRISPR/Cas9 and MMEJ/SSA in *A. oryzae*.

2. Materials and methods

2.1. Strains and medium

Escherichia coli strain DH5α (Takara Bio, Shiga, Japan) was used for plasmid propagation. It was cultured according to a standard protocol.[12] The *A. oryzae* strains RIB40 and RIB40Δ*ligD*[4] were used as host strains for the transformation assay to determine knock-in efficiency. For spore formation in *A. oryzae*, potato dextrose agar (PDA) medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 3% NaCl was used. The spores were harvested after incubation at 30 °C for 7 days by scraping with 0.1% Tween 80. For *A. oryzae* transformation, a glucose-peptone-yeast extract (GPY) medium was used for pre-culture of the fungi; it had the following composition: 20 g/L glucose, 10 g/L polypeptone (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), and 5 g/L yeast extract (Becton, Dickinson and Company, NJ, USA). Czapek–Dox medium was used as the basal medium for protoplast regeneration after transformation; it had the following composition: 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 30 g/L sucrose, 0.01 g/L $FeSO_4{\cdot}7H_2O$, and 5

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g/L $NaNO_3$.[13] The initial pH was adjusted to 5.0 using 0.1 M HCl. When necessary, sucrose (0.4–1.2 M) was added to the Czapek–Dox medium as an osmostabilizer. To select pyrithiamine-resistant strains, the Czapek–Dox medium was supplemented with pyrithiamine at a concentration of 0.1 mg/L. For the plate culture, the medium was supplemented with 10 g/L of agar.

2.2. Preparation of vectors for transformation

The fragments were amplified by polymerase chain reaction (PCR) using the genome of RIB40 as the template. KOD-FX Neo (Toyobo Co., Ltd., Osaka, Japan) was used for PCR according to the manufacturer's protocol. To evaluate the CRISPR/Cas9-mediated knock-in efficiency, the pyrithiamine resistance marker *ptrA*[14] and *sodM* promoter (*PsodM*)[15] were conjugated and inserted into the 5′ UTR region of the glucoamylase gene *glaB*, neutral protease gene *nptB*, and aspartic protease gene *pepA* (*PglaB*, *PnptB*, and *PpepA*, respectively). For vector preparation, the pPTRI-PsodM plasmid was prepared as follows. The original pPTRI plasmid (Takara Bio, Shiga, Japan) harboring the *ptrA* sequence was linearized using inverse PCR. *PsodM* amplified from the RIB40 genome was then inserted into the linearized vector using an In-Fusion cloning kit (Takara Bio, Shiga, Japan). Subsequently, the *ptrA*;*PsodM* fragment flanked by 0, 15, 30, or 50 bp homology arms (HAs) homologous to *PglaB* was prepared by PCR using pPTRI-PsodM as the template. The HA sequences are indicated in Fig. 1. Similarly, the *ptrA*;*PsodM* fragment flanked by 0, 15, or 50 bp HAs homologous to *PpepA* and *PnptB* sequence were prepared. The HA sequences are indicated in Fig. S1. For genome editing, Cas9 nuclease (EnGen Cas9 NLS; New England Biolabs Inc., MA, USA) and the sgRNA synthesis kit (EnGen sgRNA Synthesis Kit, *S. pyogenes*; New England Biolabs Inc., MA, USA) were used according to the manufacturer's instructions.[16] The CRISPR/Cas9 target sequences specific to each target region were designed and prepared as described previously.[17] The ribonucleoprotein complex was prepared using 20 μM (3.22 μg/μL) Cas9 and 20 μM sgRNA (0.65 μg/μL) dissolved in a buffer supplied by the supplier to a final volume of 5 μL (5 μg as Cas9 protein). Cas9/sgRNA ribonucleoprotein complexes were prepared immediately prior to transformation.

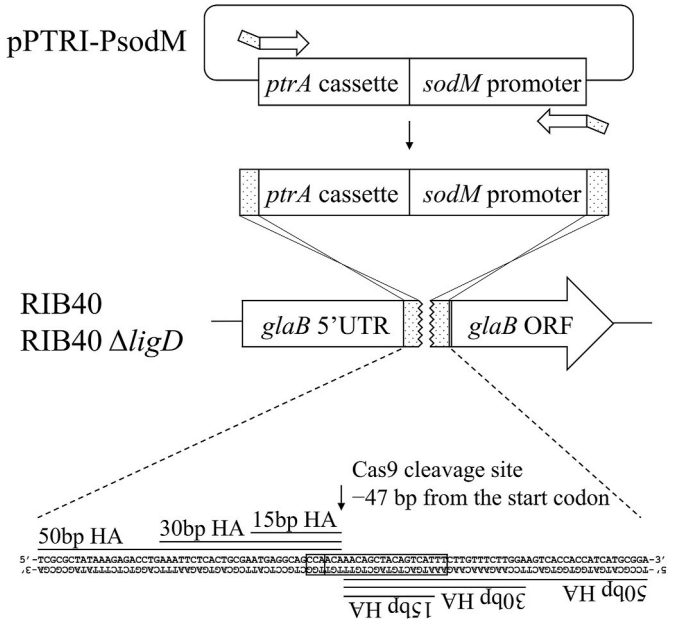

Fig. 1. Experimental design of the CRISPR/Cas9-mediated knock-in assay using the *ptrA*;*PsodM* fragment. The diagram illustrates the expected cleavage site. The 20 and 3 bp boxed sequences represent the CRISPR/Cas9 target sequence and protospacer adjacent motif, respectively; 15, 30, or 50 bp homology arms (HA) are indicated above the sequence.

2.3. Transformation procedure

Transformation was conducted using the protoplast polyethylene glycol (PEG) method. First, the spores were inoculated into 50 mL of GPY liquid medium in 300-mL Erlenmeyer flasks at a concentration of 1.0×10^6 spores/mL, followed by incubation for 16 h with rotary shaking at 120 rpm and 30 °C. The culture was then filtered using a glass filter (P250), and the mycelium was resuspended into 20 mL of an enzyme solution containing 0.8 M NaCl, 4 mg/mL Yatalase (Ozeki, Hyogo, Japan), and 2 mg/mL lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, MO, USA), followed by incubation with rotary shaking at 80 rpm and 30 °C for 3 h. The mycelium was then filtered using a glass filter (P100); the protoplast contained in the flowthrough was collected after centrifugation at 5000×*g* for 30 s and washed twice with 20 mL of 0.8 M NaCl. The protoplast was then resuspended in 50 μL of transformation solution I containing 10 mM $CaCl_2$ and 10 mM Tris-HCl at pH 7.5, yielding a density of 1×10^5 protoplasts/μL. Donor DNA (5 μg of the PCR-amplified vector) was mixed with solution I. Additionally, 5 μL (5 μg) of the Cas9/sgRNA complex was mixed with solution I for genome editing transformation. If necessary, the amount of the transformation vector and Cas9 protein was reduced to 1 μg (1/5) to prevent a reduction in experimental manageability due to an excessive number of transformants. This solution was mixed with 50 μL of solution II containing 60% (w/v) PEG 4000 (Nacalai Tesque, Kyoto, Japan), 50 mM $CaCl_2$, and 10 mM Tris-HCl at pH 7.5. Subsequently, the mixture was incubated at 20 °C for 90 min and mixed with the Czapek–Dox medium containing 1.2 M sucrose and 0.5% agar. The protoplasts with the medium were overlayered onto a pre-prepared Czapek–Dox medium containing 0.4 M sucrose and 1% agar. The medium was incubated at 30 °C for 3 days. For pyrithiamine-resistant mutant selection, each medium was supplemented with 0.1 mg/L pyrithiamine, and an additional Czapek–Dox medium containing 0.5% agar was further overlayered 24 h after the transformation procedure to inhibit the growth of background colonies. The regenerated colonies were picked, transferred to the PDA medium, and incubated at 30 °C for 5 days for spore formation and further investigation.

2.4. Evaluation of knock-in efficiency and confirmation of gene expression

After the genome editing of RIB40 with the *ptrA*;*PsodM* vector, the DNA sequence around the knock-in target site was further investigated through PCR amplification and sequencing analysis. To confirm the expression of glucoamylase in the *PglaB* transformant, a fermentation test was conducted. Spores of *A. oryzae* were inoculated into 40 mL of the GPY liquid medium in 100-mL Erlenmeyer flasks at a concentration of 1.0×10^6 spores/mL, followed by culturing with rotary shaking at 120 rpm and 30 °C for 40 h. The culture was then filtered using a paper filter (No. 101; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to separate the mycelium and supernatant, followed by further analysis of mRNA expression and enzyme production. Enzyme production was estimated using the glucoamylase and alpha-glucosidase assay kit (Kikkoman Corp., Tokyo, Japan) according to the manufacturer's manual, using the culture supernatant as the crude enzyme. Total RNA was extracted from the mycelia using ISOGEN (Nippon Gene, Tokyo, Japan) with DNase I treatment. *GlaB* expression was estimated using the One Step TB Green™ PrimeScript™ RT–PCR Kit (Takara Bio, Shiga, Japan). Thermal Cycler Dice (Takara Bio, Shiga, Japan) was used for real-time PCR. The expression levels were normalized to the internal standard (histone H2B gene) and shown as relative to the control strain using the $2^{-\Delta\Delta Ct}$ method.[18] The primer sequences used for real-time PCR are listed in Table S1.

2.5. Bioinformatics analysis

For bioinformatics analysis, genomic DNA was extracted from the

mycelia using ISO Plant II (Nippon Gene, Tokyo, Japan) with RNAse treatment. DNA sequencing was performed using a commercial service (Fasmac Co., Ltd., Kanagawa, Japan) with a 3730xl DNA Analyzer (Thermo Fisher Scientific, MA, USA). The primer sequences used for PCR are listed in Table S1.

2.6. Statistical analysis

The transformation assays and fermentation experiments were performed using three independent procedures. The results are shown as the mean ± standard deviation of three independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test to compare more than three groups or by the two-tailed unpaired Student's *t*-test to compare with the control. Excel Statistics 2015 for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for the calculations. Statistical significance was set at $p < 0.05$.

3. Results

3.1. CRISPR/Cas9-mediated knock-in efficiency in A. oryzae

We investigated whether the efficiency of targeted knock-in transformation can be improved by the use of CRISPR/Cas9 genome editing, wherein a recombinant Cas9 protein/sgRNA ribonucleoprotein complex and a knock-in template were simultaneously introduced. RIB40 and RIB40Δ*ligD* were used as the hosts for the assay. The *glaB* gene was used as the target, since GlaB protein production can be evaluated easily using an enzymatic assay. The genome editing target site was selected from the *PglaB* sequence, wherein the expected cleavage site was −47 bp from the start codon. A knock-in template harboring the pyrithiamine resistance marker *ptrA* and *PsodM* flanked by 0-, 15-, or 50-bp sequence homologous to *PglaB* was introduced, as shown in Fig. 1. The homology arms (HAs) that were 15 and 50 bp in size corresponded to the required lengths of MMEJ (2–20 bp) and SSA (>25 bp) reported in mammalian cells, respectively.[19] After selection based on pyrithiamine resistance, the mutants were further analyzed for the sequence around the CRISPR/Cas9 target site to confirm whether the intended knock-in had occurred. As shown in Fig. 2A, when the RIB40 wild type was used as the host, precise knock-in transformants could be obtained with the use of Cas9 protein whereas no such transformants were acquired without Cas9 protein. The number of correct transformants was higher with the 50 bp HA template, approximately six transformants per procedure. On the other hand, fewer than two transformants were acquired with 0 or 15 bp HA templates. Furthermore, we conducted the experiment with the Δ*ligD* host, which lacks an NHEJ repair pathway, to investigate the relationship of NHEJ with this knock-in transformation mechanism (Fig. 2B). When HA templates of >15 bp were used, >50 transformants were acquired per procedure; in contrast, no transformants were acquired with the 0 bp HA template. To further investigate the trend in knock-in efficiency, an additional comparison of 30 and 50 bp HA was conducted. As shown in Fig. S2, the number of transformants was higher with 50 bp HA when using the RIB40 wild type; however, no significant difference was observed when RIB40Δ*ligD* was used as the host. Finally, two additional target genes, *nptB* and *pepA*, were tested to assess the versatility of the knock-in system. As shown in Figs. S3 and 50 bp HAs were the most effective, resulting in approximately 9–20 transformants per procedure. Overall, these results show that HA lengths for targeted knock-in can be drastically shortened compared with those needed for the traditional method.

3.2. Effect of promoter knock-in

Next, to confirm the effect of integrated cassettes, the *glaB* gene expression in the precise knock-in strain was analyzed by real-time quantitative PCR. As shown in Fig. 3A, compared with the host

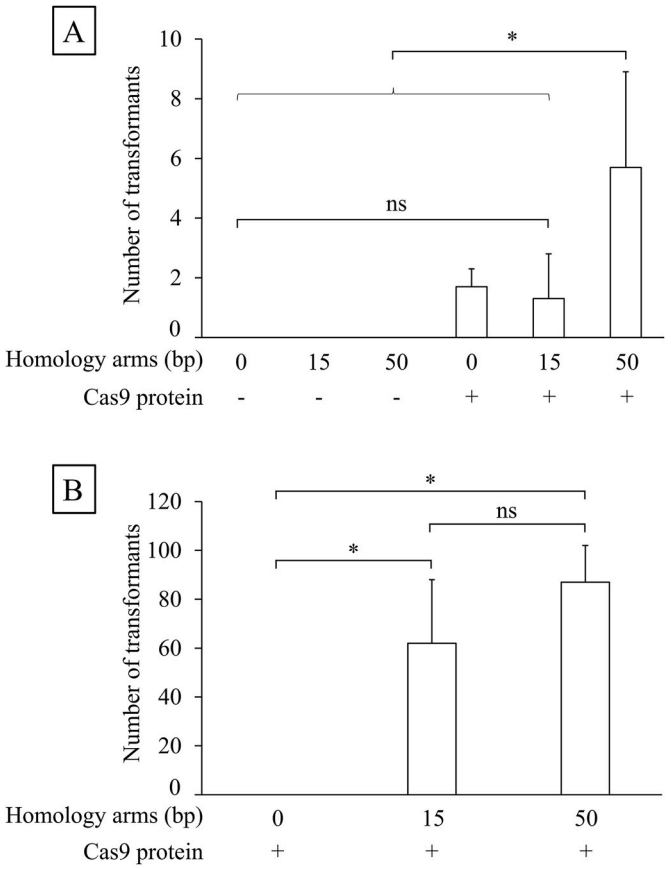

Fig. 2. Efficiency of targeted knock-in transformation. The numbers represent the acquired mutants targeting the *glaB* upstream region using RIB40 wild type (A) and RIB40Δ*ligD* (B). Amount of template DNA and Cas9 protein: 5 μg each. The numbers are presented as the mean ± standard deviation of three independent experiments. Asterisks indicate significant differences ($p < 0.05$). ns, not significant.

strains, precise *PsodM* knock-in transformants (*PsodM*:*glaB*) expressed higher levels of *glaB*. These differences were assumed to be the result of different regulations. Native *PglaB* is strongly repressed during submerged fermentation,[20] whereas *PsodM* shows strong expression under these conditions.[15] Furthermore, the increased GlaB production was confirmed using an enzymatic assay of glucoamylase activity. As shown in Fig. 3B, the enzymatic assay revealed a significant increase in glucoamylase activity in *PsodM*:*glaB* when compared with that in the host strain, thereby indicating that GlaB was functionally secreted. Taken together, our results indicate that the CRISPR/Cas9-mediated knock-in of *PsodM* effectively increased target gene expression.

4. Discussion

4.1. Repair pathway choice in A. oryzae after CRISPR/Cas9 associated DNA DSB

The present study showed that the targeted knock-in transformation of *A. oryzae* becomes easier by using the CRISPR/Cas9 system. Furthermore, some novel insights were observed. As shown in Fig. 2 and 50 bp length was sufficient to efficiently obtain precise knock-in transformants when CRISPR/Cas9 was used. It is notable that there were a few correct transformants with the 0 bp HA template when using RIB40 as the host strain, although there were no obvious homologies between the target site and template sequences (Fig. 2A). These strains are believed to result from the NHEJ repair pathway, which can join two blunt ends, as they were not observed when using the Δ*ligD* strain as the

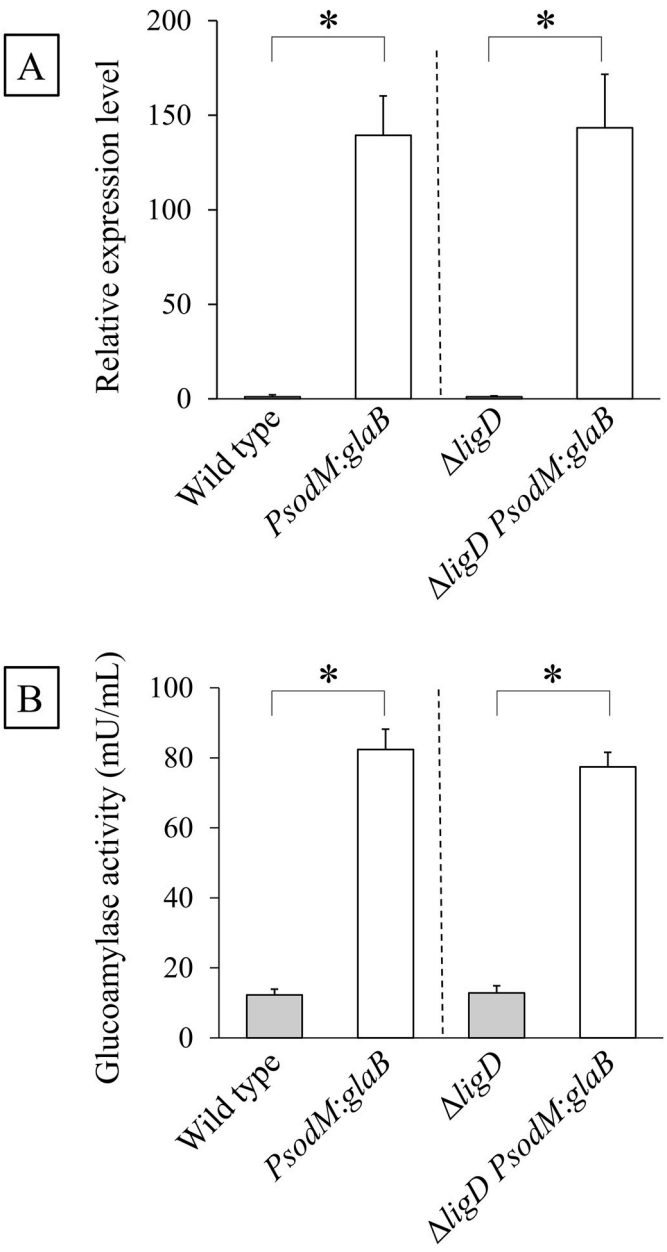

Fig. 3. Confirmation of *glaB* gene expression. (A) *GlaB* gene expression estimation using quantitative real-time PCR. The expression levels were normalized to that of histone H2B and are shown relative to the control strain. (B) Glucoamylase activity estimation. The values of the transformants were compared with those of the control strain (gray) and are shown as the mean ± standard deviation of three independent experiments. Asterisks indicate significant differences ($p < 0.05$).

host strain (Fig. 2B). The higher knock-in efficiency with 50 bp HA suggests the involvement of the SSA repair pathway. Considering the poor knock-in efficiency in the control procedure (without Cas9), SSA mediated knock-in is not efficient when the genome DNA is intact. Additionally, knock-in transformation was frequent in the Δ*ligD* background with 15–50 bp HA templates. This indicates that both MMEJ and SSA repair pathways exist independent of *ligD* and are promoted by *ligD* disruption in *A. oryzae*. This is consistent with a previous report on *A. fumigatus* using CRISPR/Cas9 and a template with 39 bp HAs.[21] However, our result is inconsistent with a previous report on *A. oryzae* using TALENs, wherein decreased MMEJ efficiency was indicated in Δ*ligD*.[8] Considering that TALENs create DNA DSB with overhangs[22] and CRISPR/Cas9 generates blunt ends,[23] the difference in cleavage site may have influenced the activity of the repair pathway. In addition, the amount of the donor DNA differed because our study used temporal DNA fragments, while the former used self-reproducing plasmid as the DNA template.[8]

4.2. CRISPR/Cas9-mediated knock-in as a novel genetic engineering method

As shown in Fig. 2 and Fig. S3, CRISPR/Cas9-mediated knock-in was effective in obtaining the targeted knock-in transformant for three different target sites. The number of acquired mutants varied depending on the target sites, likely owing to the impact of target spacer sequences on CRISPR/Cas9 cleavage efficiency.[7] Furthermore, the *PsodM* knock-in strain produced significantly more functional proteins when compared with the host strain (Fig. 3). As a targeted knock-in system, CRISPR/Cas9-mediated knock-in has some advantages over classical HR-based knock-in. First, it is useful that targeted knock-in can be accomplished without the preparation of the host. To date, most genetic engineering studies on *A. oryzae* have been conducted using a limited number of model strains such as RIB40 because of the laborious process for the preparation of an NHEJ-disruptant host strain, despite the fact that industrially important *A. oryzae* strains have a wide diversity of secretory hydrolases and secondary metabolites.[24] The preparation of an NHEJ-disruptant strain has other disadvantages in addition to the laborious process; for example, it may induce undesirable mutations in the host, because developing an NHEJ-disruptant strain requires preparing auxotroph mutants as the first step,[4,5] which is usually accomplished by ultraviolet (UV) irradiation or chemical mutagens. Furthermore, the permanent loss of NHEJ lacks a vital DNA repair pathway and may affect fitness, such as sensitivity to irradiation[25] or ethyl methanesulfonate.[4] Considering these disadvantages and the limitation of the host strain, CRISPR/Cas9-mediated knock-in is preferred from the perspective of the host strain.

Second, the laborious steps for preparing the vector can be reduced. In HR-based knock-in, constructs harboring HAs of approximately >500 bp need to be prepared for each target locus.[4] The process is usually completed by several repeated cycles of PCR, plasmid construction, and subcloning into *E. coli*, as shown in Fig. 4. In contrast, in CRISPR/Cas9-mediated knock-in, template preparations are much easier once the basic plasmid harboring a marker and knock-in purpose, such as a promoter, is prepared. For example, using the plasmid prepared in the present study, another targeted knock-in vector can be prepared with only a single PCR. Furthermore, CRISPR/Cas9-mediated knock-in will also be applicable to other systems, such as GFP knock-in. Taken together, CRISPR/Cas9-mediated knock-in is advantageous over classical HR-based methods, although further experiments using other strains are needed to confirm the versatility of this method. However, CRISPR/Cas9-mediated knock-in has a limitation in that the Cas9 target site requires the 5′-NGG-3′ protospacer adjacent motif (PAM).[23] Recently, other variations of CRISPR systems with different PAM recognition, such as CRISPR/Cas9-NG[26] and CRISPR/Cpf1,[27] have been successfully applied to yeast[28] and *A. oryzae*.[29] The use of these alternative genome editing technologies may aid in overcoming this limitation. Overall, the use of the CRISPR/Cas9-mediated knock-in system will facilitate the acquisition of targeted knock-in transformants, especially from industrially important non-model strains of *A. oryzae*. Furthermore, there are fewer studies on genome editing with SSA/MMEJ in microorganisms compared with mammalian cells. While there are relatively more reports on the model microorganism *Saccharomyces cerevisiae*, *S. cerevisiae* differs from many other fungi in its preferential use of HR repair.[30] In other fungi, although the number of reports using genome editing is increasing, studies utilizing SSA/MMEJ are limited to several species[31] such as *A. fumigatus*,[21] *Magnaporthe oryzae*,[32] and *Rhizopus microsporus*.[33] Therefore, the results obtained in this study will be beneficial for future research on filamentous fungi.

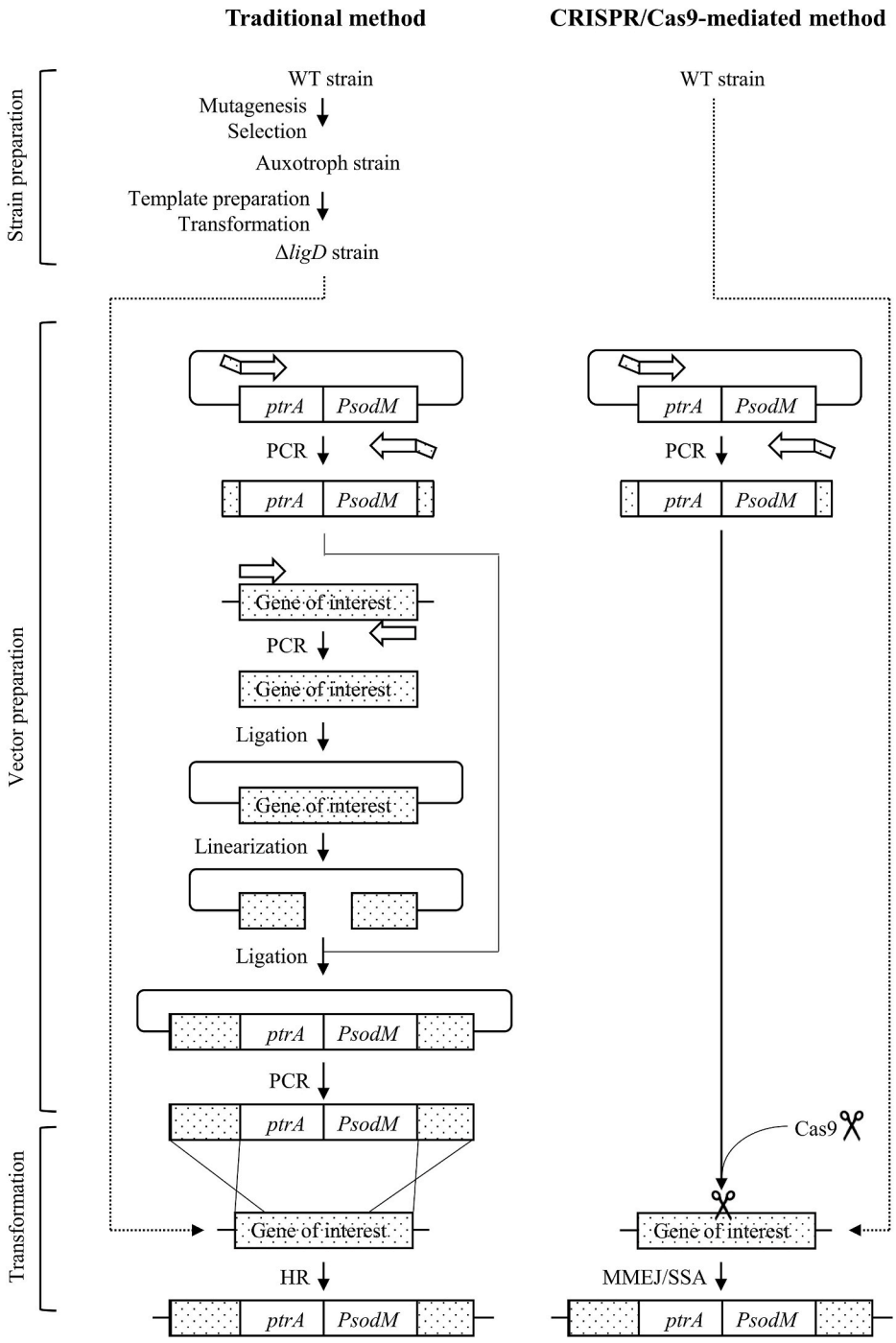

Fig. 4. Comparison of experimental flow of targeted knock-in transformation. Flow for the (left) classical method and (right) CRISPR/Cas9-mediated method.

4.3. Conclusion

In this study, we investigated a novel targeted knock-in transformation method for *A. oryzae*, an important fungus in food and industrial enzyme production. The utilization of CRISPR/Cas9 genome editing in conjunction with the MMEJ and SSA repair systems facilitated the efficient generation of targeted knock-in transformants, making the process much simpler compared with traditional methods. This approach will streamline the targeted knock-in transformation of *A. oryzae*, particularly in industrially important non-model strains.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: the authors are currently employed by Gekkeikan Sake Co., Ltd.

Acknowledgments

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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