



Forced Recycling of an AMA1-Based Genome-Editing Plasmid Allows for Efficient Multiple Gene Deletion/Integration in the Industrial Filamentous Fungus *Aspergillus oryzae*

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ABSTRACT Filamentous fungi are used for food fermentation and industrial production of recombinant proteins. They also serve as a source of secondary metabolites and are recently expected as hosts for heterologous production of useful secondary metabolites. Multiple-step genetic engineering is required to enhance industrial production involving these fungi, but traditional sequential modification of multiple genes using a limited number of selection markers is laborious. Moreover, efficient genetic engineering techniques for industrial strains have not yet been established. We have previously developed a clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9-based mutagenesis technique for the industrial filamentous fungus *Aspergillus oryzae*, enabling mutation efficiency of 10 to 20%. Here, we improved the CRISPR/Cas9 approach by including an AMA1-based autonomously replicating plasmid harboring the drug resistance marker *ptrA*. By using the improved mutagenesis technique, we successfully modified *A. oryzae* wild and industrial strains, with a mutation efficiency of 50 to 100%. Conditional expression of the *Aoace2* gene from the AMA1-based plasmid severely inhibited fungal growth. This enabled forced recycling of the plasmid, allowing repeated genome editing. Further, double mutant strains were successfully obtained with high efficiency by expressing two guide RNA molecules from the genome-editing plasmid. Cotransformation of fungal cells with the genome-editing plasmid together with a circular donor DNA enabled marker-free multiplex gene deletion/integration in *A. oryzae*. The presented repeatable marker-free genetic engineering approach for mutagenesis and gene deletion/integration will allow for efficient modification of multiple genes in industrial fungal strains, increasing their applicability.

IMPORTANCE Multiple gene modifications of specific fungal strains are required for achieving industrial-scale production of enzymes and secondary metabolites. In the present study, we developed an efficient multiple genetic engineering technique for the filamentous fungus *Aspergillus oryzae*. The approach is based on a clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system and recycling of an AMA1-based autonomous replicating plasmid. Because the plasmid harbors a drug resistance marker (*ptrA*), the approach does not require the construction of auxotrophic industrial strains prior to genome editing and allows for forced recycling of the gene-editing plasmid. The established plasmid-recycling technique involves an *Aoace2*-conditional expression cassette, whose induction severely impairs fungal growth. We used the developed genetic engineering techniques for highly efficient marker-free multiple gene deletion/integration in *A. oryzae*. The genome-editing approaches established in the present study, which enable unlimited repeatable genetic engineering, will facilitate multiple gene modification of industrially important fungal strains.

Citation Katayama T, Nakamura H, Zhang Y, Pascal A, Fujii W, Maruyama J. 2019. Forced recycling of an AMA1-based genome-editing plasmid allows for efficient multiple gene deletion/integration in the industrial filamentous fungus *Aspergillus oryzae*. *Appl Environ Microbiol* 85:e01896-18. <https://doi.org/10.1128/AEM.01896-18>.

Editor Irina S. Druzhinina, Technische Universität Wien

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Received 7 August 2018

Accepted 15 November 2018

Accepted manuscript posted online 26 November 2018

Published 23 January 2019

KEYWORDS *Aspergillus oryzae*, CRISPR/Cas9, marker-free engineering, multiplex genome editing, plasmid recycling

Filamentous fungi efficiently secrete proteins and have been used for food fermentation and industrial production of recombinant proteins (1). In addition, they serve as a source of secondary metabolites and are recently expected as hosts for heterologous production of useful secondary metabolites (2, 3). Development of transformation methods and several selection markers allowed for homologous recombination-based genetic engineering of many filamentous fungal species. Generation of deletion mutants of genes related to nonhomologous end joining (NHEJ), such as *ku70-ku80* and *ligD*, greatly enhances the engineering efficiency (4). These efforts facilitated many attempts to increase the production of proteins and secondary metabolites, e.g., overexpression of chaperone proteins and deletion of multiple protease-encoding genes for increased production of recombinant proteins (1), and introduction of multiple biosynthetic genes for the production of secondary metabolites (3), whereas multiple steps of genetic engineering involving a limited number of selection markers are required for such improvements. In addition, although the full potential of industrially exploited strains has possibly not yet been unlocked, genetic engineering of such strains remains laborious because of the difficulty in application of advanced genetic technologies in these strains.

A bacterial and archaeal immune mechanism, clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9, has been developed into a powerful genetic engineering system (5). Two components, Cas9 nuclease and a single chimeric guide RNA (sgRNA), required for the recruitment of Cas9 to a specific genomic locus, allow for the introduction of targeted DNA double-strand breaks (DSBs). This, in turn, enables genome editing, including mutagenesis by NHEJ repair or gene deletion/integration by homology-directed repair (HDR) (5). The CRISPR/Cas9 system has been applied to various organisms, such as mammalian cells, plants, fish, and yeast (5). In addition, genetic engineering using this system was also performed in industrial microbes (6).

Mutagenesis and gene deletion/integration using the CRISPR/Cas9 system have recently been reported in filamentous fungi (6, 7). In the case of mutagenesis, mutants containing deletion or insertion of nucleotide sequences of various lengths were obtained by introducing Cas9 and sgRNA expression cassettes (8–16) or a Cas9 expression cassette and synthesized sgRNA (17, 18). However, the mutation efficiencies of such systems are low: 10 to 20% in *Aspergillus oryzae* (10), 25 to 67% in *Aspergillus fumigatus* (9, 12), and 8% in *Phytophthora sojae* (14). Such efficiencies are lower than that of genetic engineering in mutants lacking NHEJ-related genes (4). In addition, genes, whose deletion affects the phenotype of transformant, such as pigment, growth, and drug resistance, have been selected as the target genes (7). Therefore, mutagenesis that does not result in phenotypic changes is probably more difficult to screen. The CRISPR/Cas9 system involving a donor DNA integrated into the recipient genome via HDR was also used for the introduction of mutation (19, 20), gene deletion (8, 12, 14, 15, 18, 21–25), and gene integration (26–28). In these studies, positively selectable markers, such as *pyrG* (*URA5*) (8, 18, 21, 22) and *amdS* (24), and drug-resistant selection markers, such as the pyriithamine resistance marker *ptrA* (19), the hygromycin resistance marker *hph* (12, 22, 25, 26), and the G418 resistance marker *neo* (*NPTII*) (14, 15, 27), were inserted into the donor DNA, resulting in higher efficiencies of genetic engineering. In contrast, Pohl et al. reported marker-free gene deletion using a donor DNA without any selection markers in *Penicillium chrysogenum* (24). Recently, marker-free introduction of a stop-codon triplet was efficiently performed by using a single-stranded oligonucleotide as the template in the NHEJ-deficient *Aspergillus* strains (20).

Genetic engineering of multiple genes requires multiplex engineering or reintroduction of sgRNA expression cassette. Simultaneous deletion of up to four genes using a single selection marker has been reported in filamentous fungi (15, 18). However, increased number of target genes leads to reduced efficiency of multiplex engineering

(15), indicating that the number of genes that can be simultaneously engineered is limited. Therefore, a method to recycle the sgRNA expression cassettes is required for efficient engineering of multiple genes.

Plasmids containing the AMA1 sequence from *Aspergillus nidulans* (29), which allows for autonomous plasmid replication, are often used for the expression of Cas9 and sgRNA (8, 12, 20–22, 24–26). Since multiple copies of the AMA1-based plasmid are present in a cell (29), this would contribute to increasing the CRISPR/Cas9-mediated mutation efficiency due to high expression of these factors. In addition, several rounds of subculturing under nonselective conditions results in the removal of the AMA1-based plasmid from transformants (30). Weyda et al. reported natural loss of the AMA1-based plasmid from *Aspergillus carbonarius* transformants by three rounds of subculturing under nonselective conditions (22). Forced recycling of the genome-editing plasmid would theoretically facilitate infinite repeatability of genetic engineering steps. Finally, drug resistance markers are preferred for genetic modifications of industrial strains, since obtaining auxotrophic mutants is laborious. Nonetheless, forced recycling of an AMA1-based plasmid containing a drug resistance marker had not been reported in filamentous fungi.

Although numerous *A. oryzae* strains are used industrially for Japanese traditional food fermentation and the production of recombinant proteins, an efficient genetic engineering method has not been established for such strains. In the present study, we performed a highly efficient mutagenesis of *A. oryzae* wild and industrial strains using AMA1-based genome-editing plasmids. We also successfully performed simultaneous double mutagenesis of these strains by expressing two sgRNAs from a single genome-editing plasmid. Further, we developed a forced recycling method for the AMA1-based genome-editing plasmid harboring the drug resistance marker and performed efficient gene deletion/integration using a donor DNA without inserting any selection markers, allowing for repeatable genetic engineering.

RESULTS

Highly efficient mutagenesis involving AMA1-based genome-editing plasmids.

We previously performed CRISPR/Cas9-mediated mutagenesis by integrating a genome-editing plasmid into the genome of *A. oryzae*; however, the mutation efficiencies of this approach were 10 to 20% (10). The strategy for mutagenesis by integrating the *cas9* and sgRNA expression cassettes into the genome also resulted in low efficiency (8%) in *P. sojae* (14). In contrast, use of the AMA1-based genome-editing plasmid allowed for efficient mutagenesis (8), possibly due to high expression of *cas9* and sgRNA from multiple copies. In the present study, we therefore constructed a new genome-editing vector, ppAsAcas9, containing the AMA1 sequence for autonomous plasmid replication to improve the efficiency of CRISPR/Cas9-mediated mutagenesis (Fig. 1A). Since truncated AMA1 is also functional (26, 29), we used half of the AMA1 sequence (half AMA1), and the pyrithiamine resistance marker *ptrA* was used for screening. Further, sgRNA expression cassettes were inserted into ppAsAcas9, yielding plasmids ppAsAC9gwA, ppAsAC9gyA, and ppAsAC9gpG, for the mutagenesis of the *wA*, *yA*, and *pyrG* genes, respectively (Fig. 1A). When the plasmids were introduced into the *A. oryzae* wild strain RIB40, we successfully obtained transformants exhibiting the expected phenotypes, i.e., white conidium formation (*wA* mutants), yellow conidium formation (*yA* mutants), and uridine/uracil auxotrophy (*pyrG* mutants) (Fig. 1B). Mutation efficiencies in RIB40 were 87.5, 100, and 55.6% for *wA*, *yA*, and *pyrG* genes, respectively (Fig. 1C). Various mutation patterns were observed, and most mutants contained short deletions of up to 26 bp (see Fig. S1 in the supplemental material). In some *yA* mutants, the regions adjacent to the target sequences were largely deleted, and large fragments from other regions of the genome were inserted. The plasmid ppAsAC9gwA for *wA* mutagenesis was also introduced into *A. oryzae* industrial strains RIB128 and RIB915, which are used for sake and soy sauce fermentation, respectively (<http://www.nrib.go.jp/data/asp/strain.html>). Transformants exhibiting white conidium formation were obtained with the mutation efficiencies of 94.1 and 58.3% for RIB128 and RIB915, respectively (Fig. 1C and D). These

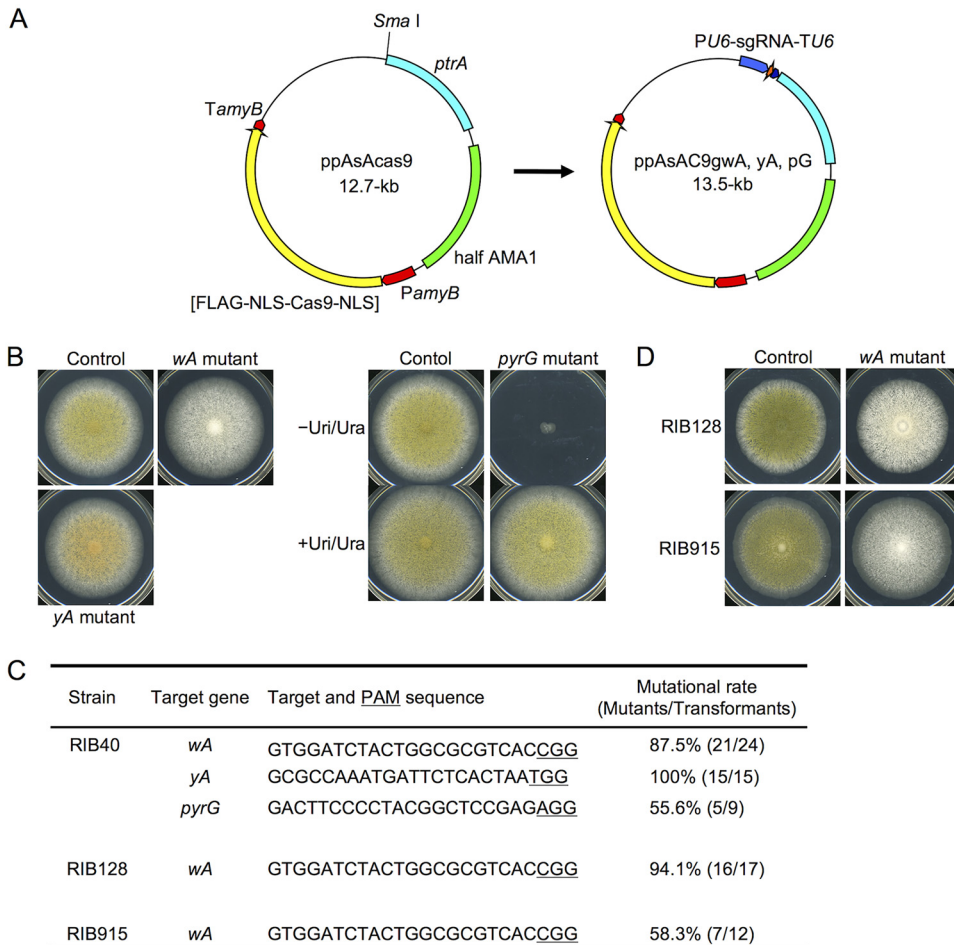


FIG 1 Mutagenesis of *A. oryzae* wild (RIB40) and industrial (RIB128 and RIB915) strains using AMA1-based genome-editing plasmids. (A) Construction of AMA1-based genome-editing plasmid. (B) Phenotypes of *wA*, *yA*, and *pyrG* mutants generated from the wild strain. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto potato dextrose (PD) agar medium for the *wA* and *yA* mutants and on PD either with uridine and uracil (+Uri/Ura) or without uridine and uracil (–Uri/Ura) for the *pyrG* mutant, followed by incubation for 5 days at 30°C . (C) Efficiencies of mutation using the AMA1-based genome-editing plasmids. (D) Phenotypes of the *wA* mutants generated from the industrial strains. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto PD agar medium, followed by incubation for 5 days at 30°C .

results indicate that the AMA1-based genome-editing plasmid enabled highly efficient mutagenesis in *A. oryzae* wild and industrial strains.

Multiple mutagenesis by recycling of genome-editing plasmids. The AMA1-based plasmid is reportedly removed from transformants by continuous subculturing on a nonselective medium (30). Efficient plasmid removal can be achieved using counterselective markers, such as *pyrG* and *niaD*, but obtaining the corresponding auxotrophic strains is laborious. Therefore, we attempted to design an efficient plasmid recycling system when a drug resistance marker is used. In a transcription factor screen, we have previously identified AO090003000678 as a gene whose deletion leads to hyperproduction of sclerotia (H. Nakamura and J. Maruyama, unpublished data). Since the orthologues of AO090003000678 are conserved in filamentous fungi, including *Aspergillus fumigatus*, of which Ace2 was identified as an orthologue to Ace2 of *Schizosaccharomyces pombe* (31), we designated AO090003000678 *Aoace2* (Fig. S2A). When *Aoace2* was expressed under the control of *amyB* promoter (*PamyB*), which is induced strongly by dextrin, induced intermediately by glucose, and repressed by glycerol (32), the harboring strain exhibited severe growth defects on a medium containing dextrin but not glycerol as the sole carbon source (Fig. S2B). As the

induction of *Aoace2* expression resulted in cell lysis (Fig. S2C), growth defect by overexpression of *Aoace2* is possibly due to its involvement in cell wall maintenance, which was suggested in *A. fumigatus* and *S. pombe* (31, 33). Thus, conditional expression of *Aoace2* from the AMA1-based plasmid could have enabled plasmid removal. To test this, the *Aoace2* expression cassette under the control of *PamyB* was inserted into the genome-editing plasmid, yielding plasmid ppAsATC9a2. Therein, the *cas9* gene was placed under the control of promoter of AO090120000080 (*Aotef1*) encoding a translation elongation factor EF-1- α (Fig. 2A). An sgRNA expression cassette was then inserted into ppAsATC9a2 (Fig. 2A).

For multiple mutagenesis by plasmid recycling, we performed three steps: first-gene mutagenesis, removal of the plasmid for first-gene mutagenesis, and second-gene mutagenesis (Fig. S3). The *wA* and *niaD* genes were selected as target genes. First, the genome-editing plasmid for *wA* mutagenesis ppAsATC9a2gwA7, containing the *Aoace2* expression cassette, was introduced into the strain RIB40 under *PamyB*-repressing conditions to retain the plasmid. As expected, a *wA* mutant RIB40wA7C9-1 that formed white conidia was obtained; a 17-bp deletion in the *wA* gene was confirmed in the mutant (Fig. 2B and C). The mutant exhibited pyrithiamine resistance, indicating that the genome-editing plasmid was retained (Fig. 2B). The mutant was then incubated under a *PamyB*-inducing condition to remove the genome-editing plasmid, yielding a strain RIB40wA7-1. The strain exhibited pyrithiamine sensitivity (Fig. 2B), and a band specific for the genome-editing plasmid was not detected by Southern blot analysis (Fig. 2D). These results indicated that the genome-editing plasmid was absent in RIB40wA7-1. In addition, the 17-bp deletion in *wA* was retained after plasmid removal (Fig. 2C). Subsequently, the genome-editing plasmid for *niaD* mutagenesis ppAsATC9a2gniaD was introduced into the plasmid-devoid *wA* mutant RIB40wA7-1. The transformant was grown under *PamyB*-inducing conditions to remove the genome-editing plasmid, yielding RIB40wAnD-1. The resulting strain did not grow on the Czapek-Dox (CD) minimal medium, in which NaNO₃ is the sole nitrogen source (Fig. 2E). Indeed, RIB40wAnD-1 harbored a 1-bp deletion in the target sequence within the *niaD* gene (Fig. 2F). We also attempted to use plasmid recycling to generate a *wA niaD* double mutant derived from the *A. oryzae* industrial strain RIB128, and we successfully obtained the double mutant containing 1-bp insertion and 1-bp deletion within the target sequences of *wA* and *niaD*, respectively (Fig. S4). These findings indicated that the recycling of the genome-editing plasmid enabled multiple gene mutagenesis in *A. oryzae* strains.

Double mutagenesis in a single transformation step using a genome-editing plasmid expressing two sgRNA molecules. In the discussion above, we presented a method for repeatable genetic engineering by recycling of a genome-editing plasmid, while construction of double mutants using this strategy relied on tandem transformation. Multiplex mutagenesis in a single transformation step would increase the efficiency of genetic engineering. When the strain RIB40 was cotransformed with two genome-editing plasmids, ppAsATC9a2gwA7 and ppAsATC9a2gniaD, for multiplex mutagenesis, only single mutants of either *wA* or *niaD*, and not *wA niaD* double mutants, were obtained (M. B. Arnaud, T. Katayama, and J. Maruyama, data not shown). Therefore, we next attempted to simultaneously express two sgRNAs from a single genome-editing plasmid. Consequently, the genome-editing plasmid pRGE5wABnD for simultaneous mutagenesis of *wA* and *niaD* was constructed (Fig. 3A) and introduced into the strain RIB40. As target sequences that included specific restriction enzyme sites were chosen in the experiment, successful mutagenesis could be detected by restriction enzyme digestion of amplified PCR products from genomic DNA (Fig. S5A). Among 22 transformants, 15 transformants were *wA niaD* double mutants (68.1%), 5 were *niaD* single mutants (22.7%), and 2 were not mutated (9.1%) (Fig. 3B and C; see also Fig. S5B). The majority of transformants exhibited the expected phenotypes: white conidium formation (*wA* mutants) and the inability to assimilate nitrate (*niaD* mutants) (Fig. S5B). As an exception, transformant 27 appeared to be the wild type, as determined by the digestion of PCR products (Fig. S5B), but it exhibited the phenotype of a *niaD* mutant

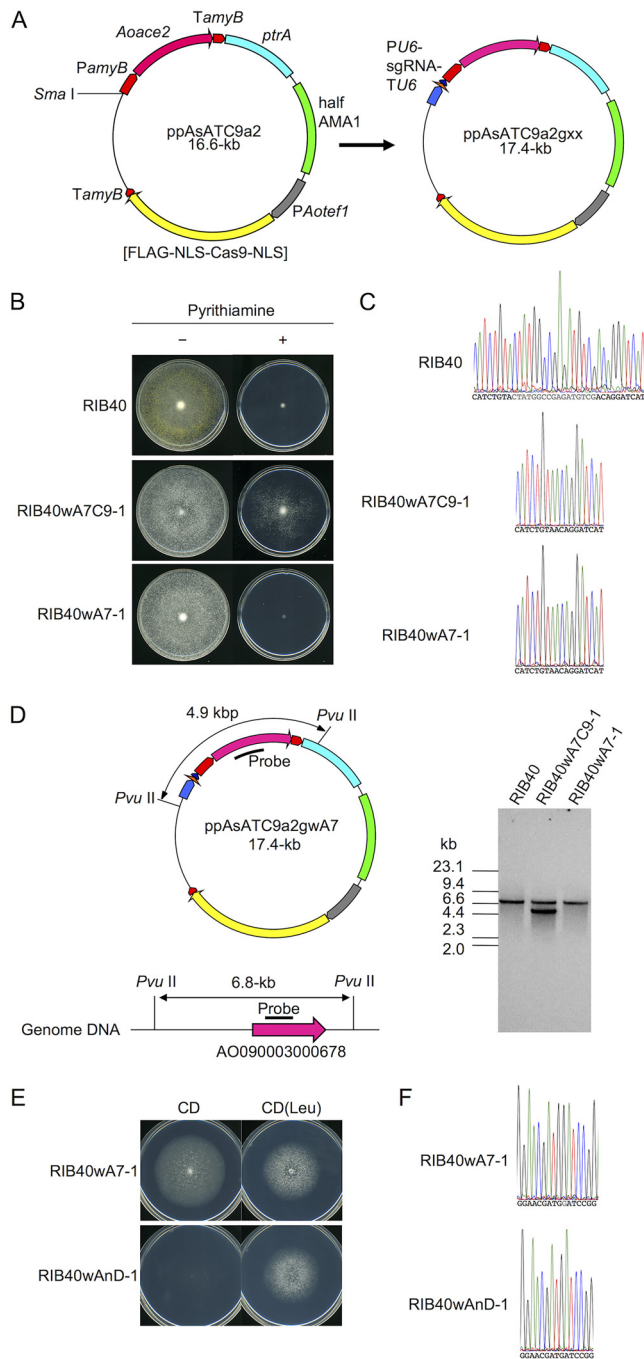


FIG 2 Generation of *wa niaD* double mutant of the *A. oryzae* wild strain RIB40 by recycling the genome-editing plasmid. (A) Construction of the genome-editing plasmid containing the *Aoace2* conditional expression cassette. (B) Pyriithiamine sensitivity test of transformants before and after plasmid removal. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto the Czapek-Dox (CD) agar medium with (+) or without (–) pyriithiamine, followed by incubation for 5 days at 30°C . (C) Nucleotide sequence analysis of the target sequence within the *wa* gene in the indicated strains. Gray letters indicate the deleted nucleotides in the generated mutants. (D) Southern blot analysis to detect the removal of the genome-editing plasmid. The indicated probe detected a 6.8-kb genomic DNA fragment in the three indicated strains and a 4.9-kb fragment of the genome-editing plasmid in the plasmid-containing strain RIB40wA7C9-1. (E) Inability to assimilate nitrate by the *wa niaD* double mutant. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto the indicated agar medium, followed by incubation for 5 days at 30°C . (F) Nucleotide sequence analysis of the target sequence within the *niaD* gene in the indicated strains. Gray letters indicate the deleted nucleotide in the generated mutants.

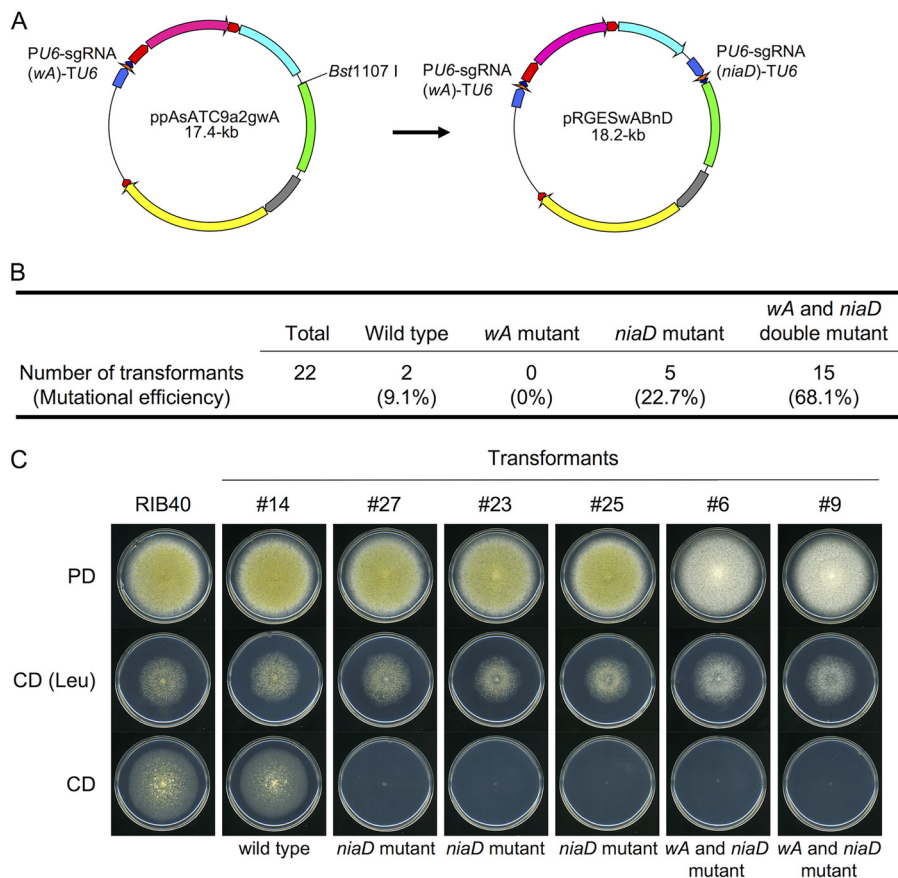


FIG 3 Multiplex mutagenesis of *wA* and *niaD* in the *A. oryzae* wild strain RIB40. (A) Construction of the genome-editing plasmid containing two sgRNA expression cassettes. (B) Efficiencies of mutation of multiplex mutagenesis of *wA* and *niaD*. (C) Phenotypes of transformants obtained by multiplex mutagenesis. The conidia of each strain were spotted onto the indicated agar medium, followed by incubation for 5 days at 30°C.

(Fig. 3C). This was probably due to a newly generated restriction site after mutagenesis at the *niaD* locus. We also investigated the feasibility of multiplex mutagenesis in the industrial strain RIB128. When the plasmid pRGESpGBwA for *wA pyrG* double mutagenesis was introduced into the strain, three of six transformants exhibited the phenotypes of a double mutant, white conidium formation and uridine/uracil auxotrophy (50%) (Fig. S6). These findings indicated that the genome-editing plasmid for simultaneous expression of two sgRNA molecules enabled efficient multiplex mutagenesis of *A. oryzae* strains.

Marker-free deletion of target genes using genome-editing plasmids and donor DNA. In filamentous fungi, the CRISPR/Cas9 system can be used for efficient gene deletion/integration by HDR using a donor DNA that contains a selection marker (7). However, multiple rounds of genetic engineering require marker-free deletion/integration. Therefore, we constructed the donor plasmid pwAupDN for the deletion of the 5' region of the *wA* gene and introduced it into the strain RIB40, together with the genome-editing plasmid pRGE-gwAup for DSB, upstream of *wA* (Fig. 4A). Since circular donor DNA is more frequently integrated into the target locus than linear donor DNA (8), we used circular donor DNA. In all 10 transformants, the 5' region of *wA* was deleted and white conidium formation was observed (Fig. 4A to C). We also constructed the donor plasmid pnDdownDN for the deletion of the 3' region of the *niaD* gene and introduced it into the strain RIB40 together with the genome-editing plasmid pRGE-gnDdown for DSB downstream of *niaD* (Fig. S7A). In all 10 transformants, the 3' region of *niaD* was deleted, and inability to assimilate nitrate was

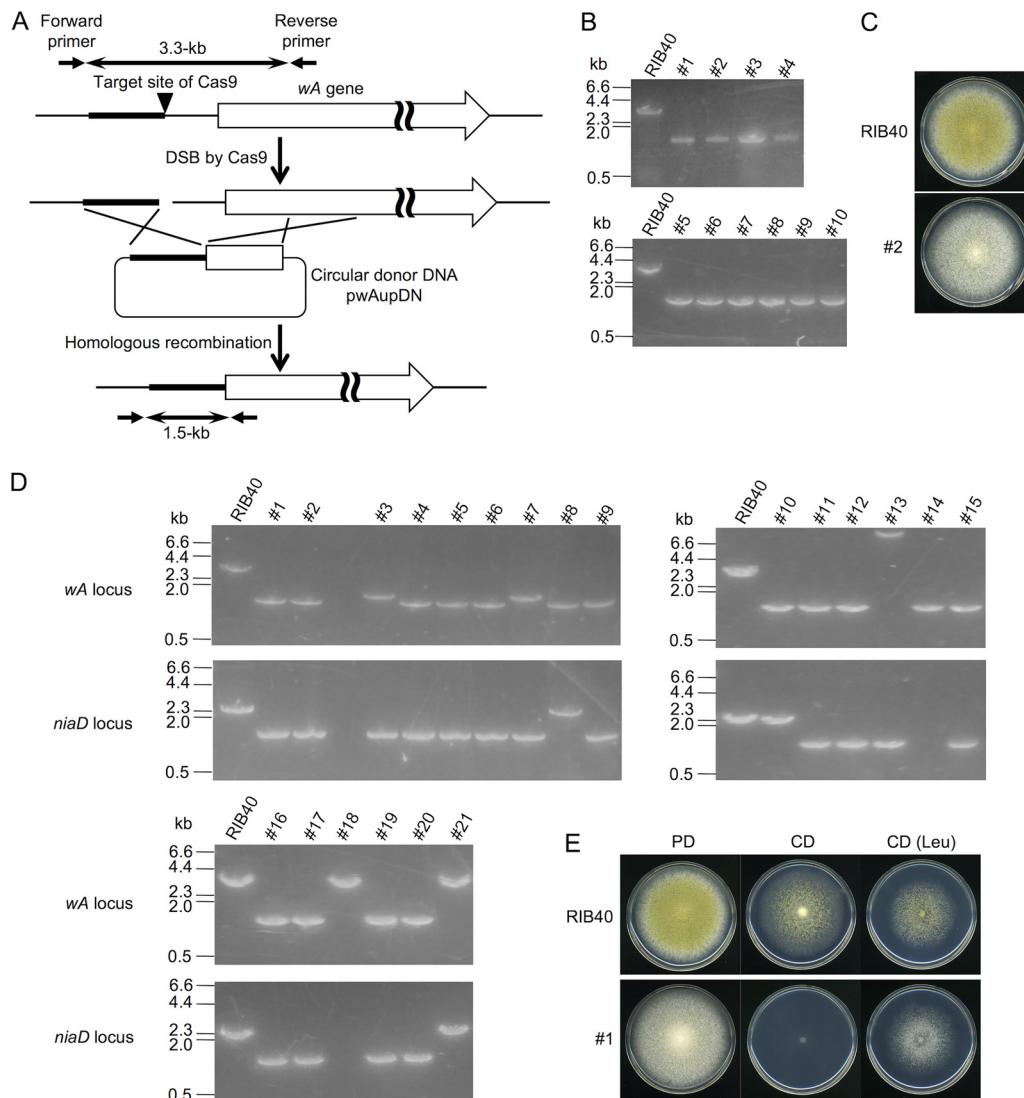


FIG 4 Gene deletion in the *A. oryzae* wild strain RIB40 by cotransformation with a genome-editing plasmid, together with a circular donor DNA. (A) Strategy for deletion of the 5' region of the *wA* gene by using the CRISPR/Cas9 system. (B) PCR analysis of genomic DNA using the primers indicated in panel A for partial deletion of *wA*. (C) Phenotype of transformants resulting from partial deletion of *wA*. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto PD agar medium, followed by incubation for 5 days at 30°C . (D) PCR analysis of genomic DNA using the primers indicated in panel A and Fig. S7A for double deletion of *wA* and *niaD*. (E) Phenotypes of transformants resulting from double deletion of *wA* and *niaD*. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto the indicated agar medium, followed by incubation for 5 days at 30°C .

observed (Fig. S7B and C). These results indicate that cointroduction of donor and genome-editing plasmids enabled efficient marker-free gene deletion in *A. oryzae* strains.

In addition, for double deletion, we constructed a genome-editing plasmid expressing two sgRNAs, pRGE-gwAupgnDdown, for DSB upstream of *wA* and downstream of *niaD*. The genome-editing plasmid was then introduced, together with the two donor plasmids, *pwAupDN* and *pnDdownDN*, into the strain RIB40. Both the 5' region of *wA* and the 3' region of *niaD* were deleted in 13 of 21 transformants (61.9%) (Fig. 4D). The selected transformants exhibited the expected phenotypes, i.e., white conidium formation and inability to assimilate nitrate (Fig. 4E). These results indicate that marker-free double gene deletion can be efficiently performed in *A. oryzae*.

Marker-free integration of exogenous genes into the genome. To integrate exogenous genes into the genome, we inserted the EGFP expression cassette under the

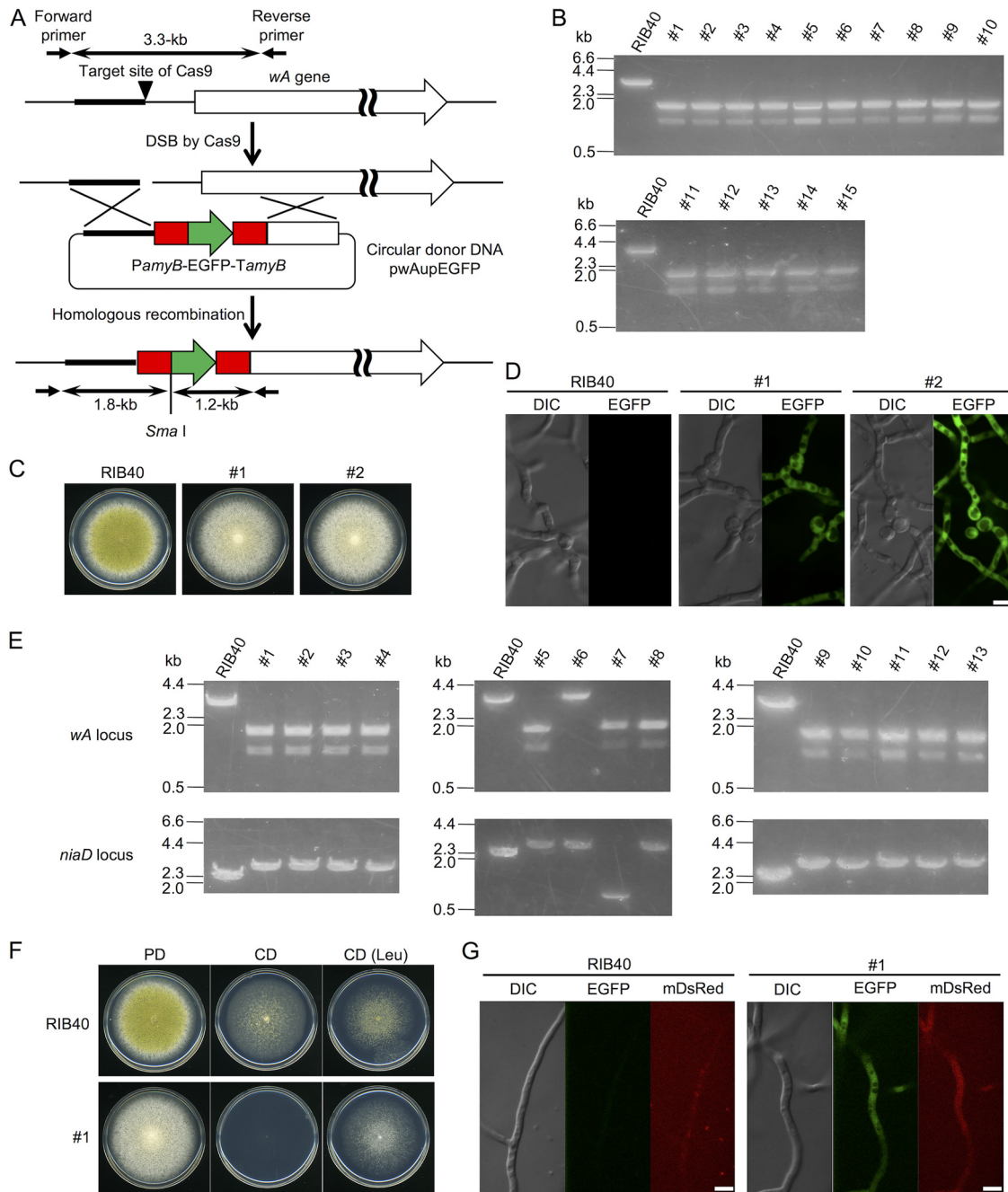


FIG 5 Gene integration in the *A. oryzae* wild strain RIB40 by cotransformation with the genome-editing plasmid together with a circular donor DNA. (A) Strategy for integration of the EGFP expression cassette into the *wA* locus by using the CRISPR/Cas9 system. (B) PCR analysis of genomic DNA and restriction enzyme digestion analysis of the integration of the EGFP expression cassette at the *wA* locus. Amplified DNA fragments using the primers indicated in panel A were digested with *Sma*I and analyzed by electrophoresis. (C) Phenotype of transformants resulting from gene integration. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto the PD medium, followed by incubation for 5 days at 30°C . (D) Fluorescent microscopic analysis of transformants expressing EGFP. Scale bar, $10 \mu\text{m}$. (E) PCR analysis of genomic DNA using the primers indicated in panel A and in Fig. S8A for double integration of the EGFP and mDsRed expression cassettes into the *wA* and the *niaD* loci, respectively. (F) Phenotype of transformants resulting from the double integration. A conidial suspension (10^4 conidia in $5 \mu\text{l}$) of each strain was spotted onto the indicated agar medium, followed by incubation for 5 days at 30°C . (G) Fluorescent microscopic analysis of transformants expressing EGFP and mDsRed. Scale bar, $10 \mu\text{m}$.

control of *PamyB* into plasmid *pwAupDN*, yielding the donor plasmid *pwAupEGFP*, and introduced it together with the genome-editing plasmid *pRGE-gwAup* into the strain RIB40. In all 15 transformants, the EGFP expression cassette was integrated into the *wA* locus (Fig. 5A and B). The selected transformants exhibited white conidium formation

and cytoplasmic enhanced green fluorescent protein (EGFP) fluorescence (Fig. 5C and D). We also constructed the donor plasmid pNDdownDR for the expression of mDsRed under the control of *PamyB* at the *niaD* locus and introduced it, together with the genome-editing plasmid pRGE-gnDdown, into strain RIB40. In all 10 transformants, the mDsRed expression cassette was integrated into the *niaD* locus (Fig. S8A and B). The selected transformants were unable to assimilate nitrate and exhibited cytoplasmic mDsRed fluorescence (Fig. S8C and D). These results demonstrated that cotransformation with donor and genome-editing plasmids enabled efficient integration of exogenous genes at the target locus.

For double integration of exogenous DNA sequences into the *wA* and *niaD* loci, we cotransformed the strain RIB40 with the genome-editing plasmid pRGE-gwAup-gnDdown and two donor plasmids, pwAupEGFP and pNDdownDR. Integration of both fragments was confirmed in 11 of 13 transformants (84.6%) (Fig. 5E). The selected transformants exhibited the expected phenotypes, white conidium formation and inability to assimilate nitrate (Fig. 5F), and cytoplasmic EGFP and mDsRed fluorescence (Fig. 5G). These observations indicated that marker-free double gene integration of exogenous genes was indeed efficiently performed in *A. oryzae*.

To investigate whether the amount of a donor plasmid influences its integration pattern into the genome, we cointroduced 1 μg of genome-editing plasmid along with with 2 or 3 μg of donor plasmid into strain RIB40. In all transformants, a single band was detected by Southern blot analysis (Fig. S9A and B), indicating that the donor DNA was integrated only into the target locus. A single copy was integrated in four and two of five transformants with 2 and 3 μg of donor plasmid, respectively, containing the EGFP expression cassette (Fig. S9A) and in two and four of five transformants with 2 and 3 μg of donor plasmid, respectively, containing the mDsRed expression cassette (Fig. S9B). These results suggest that the donor plasmid amounts tested do not affect the ratio of single-copy integration. In the transformants, except for those with single-copy integration, longer DNA fragments were detected (Fig. S9A and B), and multiple-copy integration of the donor DNA was demonstrated by quantitative PCR (Fig. S9C and D), indicating that multiple donor DNAs were tandemly integrated. In the experiment using 3 μg of donor plasmid, we obtained transformants containing multiple copies of the donor DNA, e.g., 5 copies of EGFP expression cassette and 30 copies of mDsRed expression cassette (Fig. S9C and D). In accordance, the higher production level of EGFP was detected in the transformant containing five copies of EGFP expression cassette (Fig. S9E). Hence, increased amount of multiple-copy integration of donor DNA possibly enhances the heterologous protein production.

DISCUSSION

Filamentous fungi are used for the production of industrially desirable enzymes and are expected as hosts for heterologous production of useful secondary metabolites (1, 3). Although multistep genetic engineering of recipient strains is required for efficient production of these enzymes and compounds, sequential (single) engineering of multiple genes using traditional methods is time-consuming. Previously, we developed a CRISPR/Cas9 approach for the mutagenesis of *A. oryzae*, but the efficiency of the approach was low (10). In the present study, we improved the mutation efficiency of *A. oryzae* wild and industrial strains up to 50 to 100% using autonomous replicating genome-editing plasmids harboring half AMA1 (Fig. 1C). The improved mutation efficiency was probably associated with the increased expression of Cas9 and sgRNA because the AMA1-bearing plasmid is present in multiple copies per cell (29).

For repeatable mutagenesis, we inserted the *Aoace2* gene conditionally controlled by *PamyB* into the genome-editing plasmid (Fig. 2A) and successfully removed the plasmid from the resulting mutants (Fig. 2B and D). It was previously reported that a genome-editing plasmid harboring AMA1 was removed from *A. carbonarius* transformants by continuous subculturing in the absence of selective pressure, but further genetic engineering was not performed (22). Therefore, it was unclear whether the plasmid was completely removed. Forced recycling of the AMA1-based plasmid con-

taining a drug resistance marker had not been reported in filamentous fungi. We performed further mutagenesis and obtained double mutants (Fig. 2E and F), indicating that the constructed genome-editing plasmid that harbored the *Aoace2* expression cassette was indeed removed. In addition, since the overexpression of *Aoace2* under the control of *PamyB* led to a severe growth defect of the harboring strain (Fig. S2), induction of *Aoace2* enabled complete removal of the plasmid (Fig. 2D). We also demonstrated that plasmid recycling based on *Aoace2* expression was feasible in *A. oryzae* industrial strain RIB128 (Fig. S4). Therefore, the genome-editing plasmid with conditionally expressed *Aoace2* allowed for repeatable genetic engineering of *A. oryzae* industrial strains even if no auxotrophic strains are generated.

To perform simultaneous double mutagenesis, we inserted two sgRNA expression cassettes into a single genome-editing plasmid. This resulted in the mutation efficiencies of 68.1% for *wA niaD* double mutagenesis in the wild strain RIB40 and 50% for *wA pyrG* double mutagenesis in the industrial strain RIB128 (Fig. 3A and B; see also Fig. S6). Simultaneous deletion of up to four genes by HDR using individual donor DNAs with a single selection marker has been reported (15, 18). However, increased number of target genes results in reduced homologous recombination efficiency (15), and therefore the number of simultaneously modifiable genes is thought to be limited in filamentous fungi. In contrast, the genome-editing plasmids used in double mutagenesis in the present study are recyclable since they harbor the *Aoace2* expression cassette (Fig. 3A; see also Fig. S6A).

In most previous attempts of CRISPR/Cas9-mediated HDR-based engineering in filamentous fungi, the selection markers were inserted into the donor DNAs, resulting in highly efficient genetic engineering. Recently, the marker-free HDR-based mutagenesis by using a single-stranded oligonucleotide as the template was performed with high efficiency in NHEJ-deficient *Aspergillus* strains (20). In the present study, we succeeded in efficient marker-free gene deletion/integration by cointroduction of genome-editing plasmids and circular donor DNA even in the *A. oryzae* strain with a functional NHEJ pathway (Fig. 4; see also Fig. S7). In addition, we also succeeded simultaneous double gene deletion without inserting any selection markers into the target loci (Fig. 4; see also Fig. S7). These findings indicate that marker-free genetic engineering can indeed be efficiently performed in filamentous fungi with functional NHEJ pathway. Furthermore, if the drug-resistant marker is used, the genetic engineering method developed in this study can be directly applied to filamentous fungal industrial strains without any NHEJ deficiency and auxotrophies. Many strategies for increased production of recombinant proteins using filamentous fungi have been reported (1). Among these, deletion of protease-encoding genes is an effective strategy for some *Aspergillus* species (34–36), and combinatorial deletion of up to 10 genes enhances recombinant protein production (35, 37, 38). For multiple gene deletion, the *pyrG* selection marker was recycled as the selected multiple genes were deleted one at a time in the NHEJ-deficient strain (37, 38). Consequently, generation of an NHEJ-deficient strain and multiple deletion mutants was time-consuming. The repeated multiplex gene deletion technique developed in the present study will facilitate generation of multiple gene deletion mutants in filamentous fungi.

We also demonstrated that gene integration could be efficiently performed, by inserting the EGFP and mDsRed expression cassettes into the donor DNAs without any selection marker (Fig. 5; see also Fig. S8). Filamentous fungi are used for the production of secondary metabolites such as antibiotics (2, 3). Since multiple enzymes, which are often encoded in a gene cluster on the genome, are related to a biosynthetic pathway of secondary metabolites (39), introduction of many genes is required for heterologous production in filamentous fungi (40). However, it is difficult to introduce many genes using the limited selection markers. Therefore, the technique for repeated marker-free gene integration developed in the present study would facilitate efficient generation of strains producing various secondary metabolites.

In conclusion, in the present study, we developed highly efficient techniques of CRISPR/Cas9-mediated genetic engineering, i.e., mutagenesis and gene deletion/inte-

TABLE 1 *A. oryzae* strains used in this study

Strain	Genotype or description	Source or reference
RIB40	Wild strain	45
RIB128	Industrial strain for sake fermentation	NRIB ^a
RIB915	Industrial strain for soy sauce fermentation	NRIB
SID1	<i>niaD</i> ⁻ <i>sC</i> ⁻ <i>adeA</i> ⁻ <i>argB</i> :: <i>adeA</i> ⁻ Δ <i>ligD</i> :: <i>argB</i> Δ <i>pyrG</i> :: <i>adeA</i> pgEpG[<i>pyrG</i>] pNR10[<i>niaD</i>]	46
NSID1	<i>niaD</i> ⁻ <i>sC</i> ⁻ <i>adeA</i> ⁻ <i>argB</i> :: <i>adeA</i> ⁻ Δ <i>ligD</i> :: <i>argB</i> Δ <i>pyrG</i> :: <i>adeA</i> pgEpG[<i>pyrG</i>]	47
SID-UtAoace2	<i>niaD</i> ⁻ :: <i>niaD</i> - <i>PamyB</i> - <i>Aoace2</i> - <i>TamyB</i> <i>sC</i> ⁻ <i>adeA</i> ⁻ Δ <i>argB</i> :: <i>adeA</i> ⁻ Δ <i>ligD</i> :: <i>argB</i> Δ <i>pyrG</i> :: <i>adeA</i> pgEpG[<i>pyrG</i>]	This study
RIB40wA7C9-1	<i>wA1</i> ppAsATC9a2gwA7	This study
RIB40wA7-1	<i>wA1</i>	This study
RIB40wAnD-1	<i>wA1 niaD1</i>	This study
RIB128wA7C9-1	<i>wA2</i> ppAsATC9a2gwA7	This study
RIB128wA7-1	<i>wA2</i>	This study
RIB128wAnD-1	<i>wA2 niaD1</i>	This study

^aNRIB, National Research Institute of Brewing, Higashihiroshima, Hiroshima, Japan.

gration, and for repeatable genetic engineering by plasmid recycling in *A. oryzae* wild and industrial strains. Theoretically, the combination of these techniques would allow infinite rounds of genetic engineering. Since, in various filamentous fungi, including not only *Aspergillus* species but also *P. chrysogenum*, *Talaromyces atrovirens*, *Trichoderma reesei*, and *Gaemannomyces graminis*, the AMA1 sequence was shown to be functional (8, 24, 25, 41, 42) and orthologues of *Aoace2* are also conserved (Fig. S2A) (31), repeatable genetic engineering approaches developed in the present study can be applied to other filamentous fungal species. These techniques can be used for molecular breeding of high-level heterologous production of proteins and secondary metabolites in filamentous fungi.

MATERIALS AND METHODS

***A. oryzae* strains, growth conditions, and transformation.** *A. oryzae* strains used in the present study are listed in Table 1. *A. oryzae* strains were grown in CD medium (0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, and 2% glucose [pH 5.5]) and PD medium (Nissui, Tokyo, Japan). GP medium (0.5% yeast extract, 1% Hipolypeptone [Fujifilm Wako Pure Chemical Corporation, Osaka, Japan], 2% glucose, 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O) was used for preculture during transformation. To grow the *pyrG* mutants, the CD medium was supplemented with 0.5% uridine and 0.2% uracil. CD(Leu) medium containing 10 mM leucine instead of NaNO₃ as the sole nitrogen source was used to grow the *niaD* mutants. For microscopic analysis, strains were grown in the CD medium containing 1% Casamino Acids. For the induction and repression of *PamyB*, 2% dextrin and 2% glycerol, respectively, instead of glucose were included in the CD medium as the sole carbon source. To repress *PamyB*, CD medium containing 1.2 M sorbitol was also used.

Transformation of *A. oryzae* strains. Transformation of *A. oryzae* was performed as previously described, with some modifications (43). The GP medium was used for preculture to prepare protoplasts. To select the transformants, 0.1 μg/ml pyrithiamine was added to the medium. The transformants by using the genome-editing plasmids without the *Aoace2* expression cassette were subcultured twice on the CD or CD(Leu) medium containing pyrithiamine. The DNA fragment around the target sequence was amplified by genomic PCR, and the DNA sequence was analyzed by nucleotide sequencing. The transformants by using the genome-editing plasmids bearing the *Aoace2* expression cassette were subcultured once on the CD or CD(Leu) medium containing 1.2 M sorbitol and pyrithiamine. They were further shifted onto the CD or CD(Leu) containing dextrin as the sole carbon source to remove the genome-editing plasmid, and then the DNA fragment around the target sequence was amplified by genomic PCR to investigate mutation and gene deletion/integration.

DNA manipulation. *Escherichia coli* DH5α was used for DNA manipulation. Primers used in this study are listed in Table 2. The PCR (PCR) was performed using PrimesSTAR HS DNA polymerase (TaKaRa, Otsu, Japan). An In-Fusion HD cloning kit (Clontech, Palo Alto, CA) was used for plasmid construction. Plasmids were extracted by the alkaline lysis method or using a plasmid midikit (Qiagen, Hilden, Germany). Nucleotide sequencing analysis of target genes was performed commercially by Fasmac Co., Ltd.

Construction of autonomously replicating plasmids for genome editing. The autonomously replicating plasmid ppAsAcas9 was constructed as follows: a DNA fragment containing the *ptrA* marker and a half part of AMA1 was amplified from the plasmid pPTRII (TaKaRa) using a primer set, 19IF-*ptrA*-F and 19IF-mAMA1-R, and was ligated with BamHI-digested pUC19 (TaKaRa), yielding ppAsAMA. A DNA fragment containing the *amyB* promoter, *cas9*, and the *amyB* terminator was amplified from pUNAFNC9gwA1 (10) using a primer set, *amyB*(p)-F and *amyB*(t)-R, and was ligated with HindIII-digested ppAsA, yielding ppAsAcas9. The sgRNA expression cassettes were then inserted into ppAsAcas9 as described in the supplemental material.

Construction of recycling plasmids for genome editing. The plasmid pUtNaa2N for *Aoace2*-conditional expression was constructed as follows: a DNA fragment containing the 3' region and the

TABLE 2 Primers used in this study

Primer	Sequence (5'–3')
19IF-ptrA-F	CGGTACCCGGGATCGGGCAATTGATTACGGGAT
19IF-mAMA1-R	CGACTCTAGAGGATCCTGCAGATTTTGACCTAGTTA
amyB(p)-F	GCAGGCATGCAAGCTTCATGGTGTGTTGATCATTITA
amyB(t)-R	TGATTACGCCAAGCTGGATCCCTTCTATAATAGACTAGCG
SmallF1-PU6F	GGTCGAGCTCGGTACCCCTTTAATGCCGGCTCATTCAAC
SmallF2-TU6-R	GGAATTGCCCGATCCCCCAGCAGCTCTATATCACGTGACG
Ptef-FNC9F	AAAGTTCGCACCACCTTCAAATGGATTACAAGGATGACGACG
IF-Ptef1F	GCAGGCATGCAAGCTGTAATCGCCCCGTTTGGGTTT
Ptef1R	TTTGAAGGTGGTGCGAACCTT
pUC19-niaD3_F	CGGTACCCGGGATCTAGAGCGCGCCGCAAAGGCAAATGTACGC
niaD3-PaB_R	CATGGTCATAGCTGTTCTGCACCAGCTCTTCTGGAGTT
niaDd-TaB_F	CCTGCAGATAATCTCCTGACCGGTGGAGCTGTTTTCGA
pUC19nDAADnD_R	AGGTGACTCTAGAGGCGGCCCTACGCCTGCTTAAAC
TaB-PaB_F	CCCGGGGATCTGTAGTAGAGGGTGGAGAGTATATGATGG
TaB-niaDd_R	TCGAAAACAAGCTCCACCAGGTCAGGAGATTATCTGCAGG
PaB-niaD3_F	AACTCCAAGAAGAGCTGGTGCAGGAAACAGATGACCATG
PaB-TaB_R	CCATCATATACTCTCCACCCTCTACTACAGATCCCCGGG
pUNA'Aoace2ORF_F	TCGAGCTCGGTACCCATGTTATCGAACCCACATCGCAATCT
pUNA'Aoace2ORF+TGA_R	CTACTACAGATCCCCGCTACTCCAAGCCGCTTCCCTTGTC
Aoace2-mR	ATGGACCCGCGCTCAAGATTAAGGCTGTC
Aoace2-mF	TCTTGACGCGGGTCCATGCCTCTATTA
sApAlf-PamyB	TCGAGCTCGGTACCCGGGTCTGGTGTGTTGATCATTITA
sApAlf-TamyB	AATTGCCCGATCCCCGTCAGGAGATTATCTGCAGGA
pAsATC9a2-TU6-R	AAAACACCATGACCCAGCAGCTCTATATCACGTGACG
gniaD-TU6F	GTACCATATTGGAAACGATGGTTCGTTTTAGAGCTAGAAATAGCAAGTTAAA
gniaD-PU6R	GATCCATCGTCCAATATGGTACACTTGTCTTCTTTACAATGATTTATTT
Bst-PU6F	AGCTTATTTTTGTATAATGCCGGCTCATTCAAAAC
Bst-TU6R	TATCAAAAACAGTAAGCAGCTCTATATCACGTGACG
TU6-gwA7F	GTAATATGGCCGAGATGTCGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
PU6-gwA7R	GTCGACATCTCGGCATAGTACACTTGTCTTCTTTACAATGATTTATTTACCTTCAAAG
gnD-TU6F	GTGCTAATGTATCAAGGACTTGTGTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
gnD-PU6R	AAGTCCTTGATACATTAGCACACTTGTCTTCTTTACAATGATTTATTTACCTTCAAAG
gwAup-PU6-R	TTCTAGCTCTAAAACCTGGTGCAGCTGTCGTTCCACTTGTCTTCTTTACAATGATTTATTT
19IF-wADN-F	TCGAGCTCGGTACCCGCCCATATCATTACAAG
wADN-5R	ACTTCTCCCGGTACAGTGTCTGTTCCCATGC
wADN-3F	CACGTGACCCGGGAGAAAGTGACCGATGCC
19IFwADN-R	CTCTAGAGGATCCCCGCGAATGATTGGTATAGGC
19IF-nDdownDN5F	TCGAGCTCGGTACCCGGTGGCAGCATGAGCGGTAT
nDdownDN5R	ACCAGCTCCCGGGCGGATCCATCGTCCAATA
nDdownDN3F	GATCCGGCCCCGGAGCTGGTGGGCTGGAGCTT
19IF-nDdownDN3R	CTCTAGAGGATCCCCGAGAGCGGCGAGCGAAAT
PamyB-C-linker-F2	TCGAGCTCGGTACCCGGGACCCAGCTTCTTGTACAAAGTG
Alf-cEGFPR	CTACTACAGATCCCTTACTTGTACAGCTGTCCATGC
wADN-PamyBF	AACGACACGTGACCCCTTTGATCATTITAAATTTTATATGGC
wADN-TamyBR	CGGTCACTTCTCCCGATCTTCTTCTATAATAGACTAGCG
TamyB-DsRedR	CTACTACAGATCCCCCTACAGGAACAGGTGGTGGC
nDdDN-PamyBF	CGATGGATCCGGCCCTTTGATCATTITAAATTTTATATGGC
nDdDN-TamyBR	ACCGCACAGCTCCCGGATCTTCTTCTATAATAGACTAGCG
wA seqF	GGGGCCACGCGGCTATCTCTT
wA seqR	GGCCTCTATCGGCTGTCCCGTAGTA
gwA sequence1	GACTGACCAGCTTGTGACG
gwA sequence2	CTGCAGCTGTAAGCTGTTCCG
niaD-GEF	CCGTATGTCTAAAGGGCACTTATTT
niaD-GER	CGCACCAAGCTCTTCTGGAGTTTTA
pyrG-GEF	GGCCCTAATCTTATCTACATGTGAC
pyrG-GER	CGTACGGGAGATTGTACGAACAGAT
pyrG_seqn	CCGTGATCAAAACTCACAT
niaD-sequence	GGTAACTGGGGAGAAAGACA
wADN-CoPF	GACGTGATGGCCAACACGACGAT
wADN-CoPR	CCTTCCGGTGTACATCGAGGCCATT
cPCR-niaDout-F	GACCTAAACACCAACTCCGCCGTTGCATAT
cPCR-niaDin-R	CAGCACATTCTGCCCCTAGACTTAGAGATT
ace2_probeF	CCCATTACCCTGCGACAAC
ace2_probeR	CCCTGCAGGCTCATGTTTTT
wA-RTF	GCGATTGCGGGATACTTTTA
wA-RTR	ATGGCAATGGATGAGGAAAG
niaD-RTF	TGGTTCATCGTGAATGGAGA
niaD-RTR	CAGAGACATCCAGACCAGCA

terminator of *niaD*, downstream of *niaD*, and the *amyB* terminator were amplified from the genome DNA of *A. oryzae* wild strain RIB40 using the primer sets pUC19-*niaD3_F* and *niaD3-PaB_R*, *niaDd-TaB-F* and pUC19nDAADnD_R, and *TaB-PaB_F* and *TaB-niaDd_R*, respectively. The *amyB* promoter was amplified from pUNA (44) using the primer set *PaB-niaD3_F* and *PaB-TaB_R*. These four fragments were fused using the primer set pUC19-*niaD3_F* and pUC19nDAADnD_R and ligated with BamHI-digested pUC19, yielding pUtNAN. The *Aoace2* ORF was amplified from the genome DNA of strain RIB40 using a primer set (pUNA'*Aoace2ORF_F* and pUNA'*Aoace2ORF+TGA_R*) and ligated with SmaI-digested pUtNAN, yielding pUtNAa2N.

The recycling plasmid ppAsATC9a2 for genome editing was constructed as follows. A DNA fragment containing *cas9* and the *amyB* terminator was amplified from the plasmid ppAsAcas9 using the primer set Ptef-FNC9F and *amyB(t)-R*. The *Aotef1* promoter was amplified from the genome DNA of the strain RIB40 using the primer set IF-Ptef1F and Ptef1R. These two DNA fragments were fused using the primer set IF-Ptef1F and *amyB(t)-R*, and the fused fragment was ligated with SmaI-digested ppAsAMA, yielding ppAsATC9. A DNA fragment containing the *amyB* promoter and the 5' half of *Aoace2* was amplified from the plasmid pUtNAa2N using the primer set sApAIF-PamyB and *Aoace2-mR*. A DNA fragment containing 3' half of *Aoace2* and the *amyB* terminator was amplified from pUtNAa2N using the primer set *Aoace2-mF* and sApAIF-TamyB. These two DNA fragments were fused using the primer set sApAIF-PamyB and sApAIF-TamyB, and the fused fragment was ligated with SmaI-digested ppAsATC9, yielding ppAsATC9a2. The sgRNA expression cassettes were then inserted into ppAsATC9a2 as described in the supplemental material.

Construction of the *Aoace2*-expressing strain. NotI-digested pUtNAa2N was introduced into the strain NSID1, yielding strain SID-UtAoace2 (Fig. S10A). The integration of the *Aoace2* expression cassette downstream of *niaD* was confirmed by PCR analysis of genome DNA (Fig. S10B).

Extraction of *A. oryzae* genome DNA and Southern blot analysis. Genomic DNA of *A. oryzae* was extracted as previously described (43). For Fig. 2D and Fig. S4C, genomic DNA was digested with PvuII, and a DNA fragment amplified from pUtNAa2N using the primer set *ace2_probeF* and *ace2_probeR* was used as a probe in Southern blot analysis. For Fig. S9A, genomic DNA was digested with BglII and DNA fragment amplified from pWAupEGFP using the primer set 19IF-wADN-F and wADN-5R. For Fig. S9B, genomic DNA was digested with EcoRV and DNA fragment amplified from pNDdownDR using the primer set 19IF-nDdownDN5F and nDdownDN5R. Southern blot analysis was performed with an ECL detection kit (GE Healthcare, IL) according to the manufacturer's instructions, and the nucleic acid bands were detected using a LAS-4000 miniEPUV luminescent image analyzer (Fuji Photo Film, Tokyo, Japan).

Fluorescence microscopy and image analysis. Conidia (1×10^4) were inoculated into 150 μ l of the CD liquid medium containing Casamino Acids in a glass-bottomed dish and incubated at 30°C for 18 h. The cultures were observed by confocal laser scanning microscopy using an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with a $\times 40$ Neofluor lens objective (1.00 numerical aperture), 488-nm (Furukawa Electric, Tokyo, Japan) and 561-nm (Melles Griot, Rochester, NY) semiconductor lasers, GFP and DsRed filters (Nippon Roper, Tokyo, Japan), a CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan), and an Andor iXon cooled digital CCD camera (Andor Technology PLC, Belfast, UK). The captured images were analyzed using Andor iQ 1.9 software (Andor Technology PLC).

Construction of plasmids, quantitative PCR, and Western blot analysis. The construction of the genome-editing plasmids containing sgRNA expression cassettes and of the donor plasmids is described in detail in the supplemental material, as well as the methods used for the quantitative PCR and Western blot analyses.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01896-18>.

SUPPLEMENTAL FILE 1, PDF file, 12.7 MB.

ACKNOWLEDGMENTS

This study was financially supported by a Grant-in-Aid for Scientific Research on Innovative Areas (17H05431) and by Grants-in-Aid for Scientific Research (18H02123 and 17K15242) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan). We thank Yasuji Koyama and Masahiro Ogawa (Noda Institute for Scientific Research, Japan) for technical support in analysis of transcription factors.

T.K., H.N., Y.Z., A.P., W.F., and J.M. conceived and designed the experiments. T.K., H.N., Y.Z., and A.P. performed the experiments. T.K., H.N., Y.Z., A.P., and J.M. analyzed the data. T.K. and J.M. wrote the manuscript. All the authors reviewed the manuscript.

There are no conflicts of interest to declare.

REFERENCES

1. Ward OP. 2012. Production of recombinant proteins by filamentous fungi. *Biotechnol Adv* 30:1119–1139. <https://doi.org/10.1016/j.biotechadv.2011.09.012>.
2. Hoffmeister D, Keller NP. 2007. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat Prod Rep* 24:393–416. <https://doi.org/10.1039/b603084j>.

3. Alberti F, Foster GD, Bailey AM. 2017. Natural products from filamentous fungi and production by heterologous expression. *Appl Microbiol Biotechnol* 101:493–500. <https://doi.org/10.1007/s00253-016-8034-2>.
4. Jiang D, Zhu W, Wang Y, Sun C, Zhang KQ, Yang J. 2013. Molecular tools for functional genomics in filamentous fungi: recent advances and new strategies. *Biotechnol Adv* 31:1562–1574. <https://doi.org/10.1016/j.biotechadv.2013.08.005>.
5. Doudna JF, Charpentier E. 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346:1258096. <https://doi.org/10.1126/science.1258096>.
6. Donohoue PD, Barrangou R, May AP. 2017. Advances in industrial biotechnology using CRISPR-Cas systems. *Trends Biotechnol* 35:167–177. <https://doi.org/10.1016/j.tibtech.2017.07.007>.
7. Shi TQ, Liu GN, Ji RY, Shi K, Song P, Ren LJ, Huang H, Ji XJ. 2017. CRISPR/Cas9-based genome editing of the filamentous fungi: the state of the art. *Appl Microbiol Biotechnol* 101:7435–7443. <https://doi.org/10.1007/s00253-017-8497-9>.
8. Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH. 2015. A CRISPR-Cas9 system for engineering of filamentous fungi. *PLoS One* 10:e0133085. <https://doi.org/10.1371/journal.pone.0133085>.
9. Fuller KK, Chen S, Loros JJ, Dunlap JC. 2015. Development of the CRISPR/Cas9 system for targeted gene disruption in *Aspergillus fumigatus*. *Eukaryot Cell* 14:1073–1080. <https://doi.org/10.1128/EC.00107-15>.
10. Katayama T, Tanaka Y, Okabe T, Nakamura H, Fujii W, Kitamoto K, Maruyama J. 2016. Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. *Biotechnol Lett* 38:637–642. <https://doi.org/10.1007/s10529-015-2015-x>.
11. Nakamura H, Katayama T, Okabe T, Iwashita K, Fujii W, Kitamoto K, Maruyama J. 2017. 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. *J Gen Appl Microbiol* 63:172–178. <https://doi.org/10.2323/jgam.2016.10.002>.
12. Zhang C, Meng X, Wei X, Lu L. 2016. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. *Fungal Genet Biol* 86:47–57. <https://doi.org/10.1016/j.fgb.2015.12.007>.
13. Wenderoth M, Pinecker C, Voss B, Fischer R. 2017. Establishment of CRISPR/Cas9 in *Alternaria alternata*. *Fungal Genet Biol* 101:55–60. <https://doi.org/10.1016/j.fgb.2017.03.001>.
14. Fang Y, Tyler BM. 2016. Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. *Mol Plant Pathol* 17:127–139. <https://doi.org/10.1111/mpp.12318>.
15. Liu Q, Gao R, Li J, Lin L, Zhao J, Sun W, Tian C. 2017. Development of a genome-editing CRISPR/Cas9 system in thermophilic fungal *Myceliophthora* species and its application to hyper-cellulase production strain engineering. *Biotechnol Biofuels* 10:1. <https://doi.org/10.1186/s13068-016-0693-9>.
16. Schuster M, Schweizer G, Reissmann S, Kahmann R. 2016. Genome editing in *Ustilago maydis* using the CRISPR-Cas9 system. *Fungal Genet Biol* 89:3–9. <https://doi.org/10.1016/j.fgb.2015.09.001>.
17. Qin H, Xiao H, Zou G, Zhou Z, Zhong JJ. 2017. CRISPR-Cas9 assisted gene disruption in the higher fungus *Ganoderma* species. *Process Biochem* 56:57–61. <https://doi.org/10.1016/j.procbio.2017.02.012>.
18. Liu R, Chen L, Jiang Y, Zhou Z, Zou G. 2015. Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. *Cell Discov* 1:15007. <https://doi.org/10.1038/celldisc.2015.7>.
19. Weber J, Valiante V, Nødvig CS, Mattern DJ, Slotkowski RA, Mortensen UH, Brakhage AA. 2017. Functional reconstruction of a fungal natural product gene cluster by advanced genome editing. *ACS Synth Biol* 6:62–68. <https://doi.org/10.1021/acssynbio.6b00203>.
20. Nødvig CS, Hoof JB, Kogle ME, Jarczyńska ZD, Lehmebeck J, Klitgaard DK, Mortensen UH. 2018. Efficient oligonucleotide-mediated CRISPR-Cas9 gene editing in aspergilli. *Fungal Genet Biol* 115:78–89. <https://doi.org/10.1016/j.fgb.2018.01.004>.
21. Kuivanen J, Wang YM, Richard P. 2016. Engineering *Aspergillus niger* for galactaric acid production: elimination of galactaric acid catabolism by using RNA sequencing and CRISPR/Cas9. *Microb Cell Fact* 15:210. <https://doi.org/10.1186/s12934-016-0613-5>.
22. Weyda I, Yang L, Vang J, Ahring B, Lübeck M, Lübeck PS. 2017. A comparison of *Agrobacterium*-mediated transformation and protoplast-mediated transformation with CRISPR-Cas9 and bipartite gene targeting substrate, as effective gene targeting tools for *Aspergillus carbonarius*. *J Microbiol Methods* 135:26–34. <https://doi.org/10.1016/j.mimet.2017.01.015>.
23. Arazoe T, Miyoshi K, Yamato T, Ogawa T, Ohsato S, Arie T, Kuwata S. 2015. Tailor-made CRISPR/Cas system for highly efficient targeted gene replacement in the rice blast fungus. *Biotechnol Bioeng* 112:2543–2549. <https://doi.org/10.1002/bit.25662>.
24. Pohl C, Kiel JAKW, Driessen AJM, Bovenberg RAL, Nygård Y. 2016. CRISPR/Cas9-based genome editing of *Penicillium chrysogenum*. *ACS Synth Biol* 5:754–764. <https://doi.org/10.1021/acssynbio.6b00082>.
25. Nielsen ML, Isbrandt T, Rasmussen KB, Thrane U, Hoof JB, Larsen TO, Mortensen UH. 2017. Genes linked to production of secondary metabolites in *Talaromyces atrovirens* revealed using CRISPR-Cas9. *PLoS One* 12:e0169712. <https://doi.org/10.1371/journal.pone.0169712>.
26. Sarkari P, Marx H, Blumhoff ML, Mattanovich D, Sauer M, Steiger MG. 2017. An efficient tool for metabolic pathway construction and gene integration in *Aspergillus niger*. *Bioresour Technol* 245:1327–1333. <https://doi.org/10.1016/j.biortech.2017.05.004>.
27. Zheng YM, Lin FL, Gao H, Zou G, Zhang JW, Wang GQ, Chen GD, Zhou ZH, Yao XS, Hu D. 2017. Development of a versatile and conventional technique for gene disruption in filamentous fungi based on CRISPR-Cas9 technology. *Sci Rep* 7:9250. <https://doi.org/10.1038/s41598-017-10052-3>.
28. Matsu-Ura T, Baek M, Kwon J, Hong C. 2015. Efficient gene editing in *Neurospora crassa* with CRISPR technology. *Fungal Biol Biotechnol* 2:4. <https://doi.org/10.1186/s40694-015-0015-1>.
29. Gems D, Johnstone IL, Clutterbuck AJ. 1991. An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. *Gene* 98:61–67. [https://doi.org/10.1016/0378-1119\(91\)90104-J](https://doi.org/10.1016/0378-1119(91)90104-J).
30. Aleksenko A, Clutterbuck AJ. 1997. Autonomous plasmid replication in *Aspergillus nidulans*: AMA1 and MATE elements. *Fungal Genet Biol* 21:373–387. <https://doi.org/10.1006/fgbi.1997.0980>.
31. Ezykowitz DE, Cunha MM, Rozental S, Solis NV, Gravelat FN, Sheppard DC, Filler SG. 2009. The *Aspergillus fumigatus* transcription factor Ace2 governs pigment production, conidiation, and virulence. *Mol Microbiol* 72:155–169. <https://doi.org/10.1111/j.1365-2958.2009.06631.x>.
32. Tada S, Gomi K, Kitamoto K, Takahashi K, Tamura G, Hara S. 1991. Construction of a fusion gene comprising the Taka-amylase A promoter and the *Escherichia coli* β -glucuronidase gene and analysis of its expression in *Aspergillus oryzae*. *Mol Gen Genet* 229:301–306. <https://doi.org/10.1007/BF00272170>.
33. Bähler J. 2005. A transcriptional pathway for cell separation in fission yeast. *Cell Cycle* 4:39–41. <https://doi.org/10.4161/cc.4.1.1336>.
34. van den Hombergh JPTW, Gelpke MDS, van de Vondervoort PJJ, Buxton FP, Visser J. 1997. Disruption of three acid proteases in *Aspergillus niger*: effect on protease spectrum, intracellular proteolysis, and degradation of target proteins. *Eur J Biochem* 247:605–613. <https://doi.org/10.1111/j.1432-1033.1997.00605.x>.
35. Jin FJ, Watanabe T, Juvvadi PR, Maruyama J, Arioka M, Kitamoto K. 2007. Double disruption of the proteinase genes, *tpaA* and *pepE*, increases the production level of human lysozyme by *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 76:1059–1068. <https://doi.org/10.1007/s00253-007-1088-4>.
36. Moralejo FJ, Cardoza RE, Gutierrez S, Lombrana M, Fierro F, Martín JF. 2002. Aspergillopepsin (*pepB*) gene of *Aspergillus awamori* by antisense RNA removal or protease removal by gene disruption results in a large increase in thaumatin production. *Appl Environ Microbiol* 68:3550–3559. <https://doi.org/10.1128/AEM.68.7.3550-3559.2002>.
37. Yoon J, Kimura S, Maruyama J, Kitamoto K. 2009. Construction of quintuple protease gene disruptant for heterologous protein production in *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 82:691–701. <https://doi.org/10.1007/s00253-008-1815-5>.
38. Yoon J, Maruyama J, Kitamoto K. 2011. Disruption of ten protease genes in the filamentous fungus *Aspergillus oryzae* highly improves production of heterologous proteins. *Appl Microbiol Biotechnol* 89:747–759. <https://doi.org/10.1007/s00253-010-2937-0>.
39. Inglis DO, Binkley J, Skrzypek MS, Arnaud MB, Cerqueira GC, Shah P, Wymore F, Wortman JR, Sherlock G. 2013. Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of *Aspergillus nidulans*, *A. fumigatus*, *A. niger*, and *A. oryzae*. *BMC Microbiol* 13:91. <https://doi.org/10.1186/1471-2180-13-91>.
40. Alberti F, Khairudin K, Venegas ER, Davies JA, Hayes PM, Willis CL, Bailey AM, Foster GD. 2017. Heterologous expression reveals the biosynthesis of the antibiotic pleuromutilin and generates bioactive semi-synthetic derivatives. *Nat Commun* 8:1831. <https://doi.org/10.1038/s41467-017-01659-1>.
41. Kubodera T, Yamashita N, Nishimura A. 2002. Transformation of *Asper-*

- gillus* sp. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. *Biosci Biotechnol Biochem* 66:404–406. <https://doi.org/10.1271/bbb.66.404>.
42. Ruiz-Díez B. 2002. Strategies for the transformation of filamentous fungi. *J Appl Microbiol* 92:189–195. <https://doi.org/10.1046/j.1365-2672.2002.01516.x>.
 43. Maruyama J, Kitamoto K. 2011. Targeted gene disruption in *koji* mold *Aspergillus oryzae*. *Methods Mol Biol* 765:447–456. https://doi.org/10.1007/978-1-61779-197-0_27.
 44. Wada R, Jin FJ, Koyama Y, Maruyama J, Kitamoto K. 2014. Efficient formation of heterokaryotic sclerotia in the filamentous fungus *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 98:325–334. <https://doi.org/10.1007/s00253-013-5314-y>.
 45. Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, et al. 2005. Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* 438: 1157–1161. <https://doi.org/10.1038/nature04300>.
 46. Nakamura H, Kikuma T, Jin FJ, Maruyama J, Kitamoto K. 2016. AoRim15 is involved in conidial stress tolerance, conidiation and sclerotia formation in the filamentous fungus *Aspergillus oryzae*. *J Biosci Bioeng* 121: 365–371. <https://doi.org/10.1016/j.jbiosc.2015.08.011>.
 47. Yoon J, Aishan T, Maruyama J, Kitamoto K. 2010. Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*. *Appl Environ Microbiol* 76:5718–5727. <https://doi.org/10.1128/AEM.03087-09>.