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

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Construction of the genome editing plasmids containing sgRNA-expression cassettes and the donor plasmids

Autonomously replicating plasmids for genome editing: The genome editing plasmids ppAsAC9gwA, ppAsAC9gyA, and ppAsAC9gpG for mutagenesis of the *wA*, *yA*, and *pyrG* genes were constructed as follows: DNA fragments containing the *U6* promoter (P*U6*), sgRNA encoding sequences, and the *U6* terminator (T*U6*) were amplified from the plasmids pUNAFNC9gwA1, pUNAFNC9gyA, and pUNAFNC9gpG (1), respectively, using a primer set, SmaIIF1-PU6F and SmaIIF2-TU6-R, and were ligated with *Sma*I-digested ppAsAcas9, yielding ppAsAC9gwA, ppAsAC9gyA, and ppAsAC9gpG, respectively.

Recycling plasmids for genome editing: The genome editing plasmid ppAsATC9a2gwA7 for mutagenesis of the *wA* gene was constructed as follows: the P*U6* was amplified from the plasmid ppAsAC9gwA using a primer set, SmaIIF1-PU6F and PU6-gwA7R. A DNA fragment containing sgRNA-encoding sequence and T*U6* was amplified from the plasmid ppAsAC9gwA using a primer set, TU6-gwA7F and pAsATC9a2-TU6-R. These DNA fragments were fused using a primer set, SmaIIF1-PU6F and pAsATC9a2-TU6-R, and the fused fragment was ligated with *Sma*I-digested ppAsATC9a2, yielding ppAsATC9a2gwA7.

The genome editing plasmid ppAsATC9a2gpG for mutagenesis of the *pyrG* gene was constructed as follows: A DNA fragments containing P*U6*, sgRNA encoding sequence, and T*U6* were amplified from the plasmid ppAsAC9gpG using a primer set, SmaIIF1-PU6F and pAsATC9a2-TU6-R, and was ligated with *Sma*I-digested ppAsATC9a2, yielding ppAsATC9a2gpG.

The genome editing plasmid ppAsATC9a2gniaD for mutagenesis of the *niaD* gene was constructed as follows: the P*U6* was amplified from the plasmid ppAsAC9gwA using a primer set, SmaIIF1-PU6F and gniaD-PU6R. A DNA fragment containing sgRNA-encoding sequence and T*U6* was amplified from ppAsAC9gwA using a primer set, gniaD-TU6F and pAsATC9a2-TU6-R. These DNA fragments were fused using a primer set, SmaIIF1-PU6F and pAsATC9a2-TU6-R, and the fused fragment was ligated with *Sma*I-digested ppAsATC9a2, yielding ppAsATC9a2gniaD.

Genome editing plasmids for double gene mutagenesis: The genome editing plasmid pRGESwABnD for double mutagenesis of the *wA* and *niaD* genes was constructed as follows: a DNA fragment containing P*U6*, sgRNA encoding sequence, and T*U6* was amplified from the plasmid ppAsATC9a2gniaD using a primer set, Bst-PU6F and Bst-TU6R, and was ligated with *Bst*1107I-digested ppAsATC9a2gwA7, yielding pRGESwABnD.

The genome editing plasmid pRGESpGBwA for double mutagenesis of the *pyrG* and *wA* genes was constructed as follows: a DNA fragment containing P*U6*, sgRNA encoding sequence, and T*U6* was amplified from the plasmid pUNAFNC9gwA1 using primer set, Bst-PU6F and Bst-TU6R, and was ligated with *Bst*1107I-digested ppAsATC9a2gpG, yielding pRGESpGBwA.

Genome editing plasmid and donor plasmid for deletion at the *wA* locus: The genome editing plasmid pRGEgwAup for DSB at the upstream of *wA* was constructed as follows: the DNA fragment containing sgRNA encoding sequence and T*U6* was amplified from the plasmid ppAsATC9a2gwA7 using a primer set, pAsAIF-gRNAF and pAsATC9a2-TU6-R, and was ligated with *Sma*I-digested ppAsATC9a2, yielding pRGE-gRT6. The P*U6* was amplified from ppAsATC9a2gwA7 using a primer

set, SmaIIF1-PU6F and gwAup-PU6-R, and was ligated with *Sma*I-digested pRGE-gRT6, yielding pRGEgwAup.

The donor plasmid pwAupDN for deletion of 5′ region of the *wA* gene was constructed as follows: the upstream region of the *wA* promoter was amplified from the genome DNA of the strain RIB40 using a primer set, 19IF-wADN-F and wADN-5R. A region within the *wA* ORF was amplified from the genome DNA of the strain RIB40 using a primer set, wADN-3F and 19IFwADN-R. These two DNA fragments were fused using a primer set, 19IF-wADN-F and 19IFwADN-R, and the fused fragment was ligated with *Sma*I-digested pUC19, yielding pwAupDN.

Genome editing plasmid and donor plasmid for deletion at the *niaD* locus: The genome editing plasmid pRGEgnDdown for DSB at the downstream of *niaD* was constructed as follows: the P*U6* was amplified from the plasmid ppAsAC9gwA using a primer set, SmaIIF1-PU6F and gnD-PU6R. A DNA fragment containing sgRNA encoding sequence and T*U6* was amplified from ppAsAC9gwA using a primer set, gnD-TU6F and pAsATC9a2-TU6-R. These DNA fragments were fused using a primer set, SmaIIF1-PU6F and pAsATC9a2-TU6-R, and the fused fragment was ligated with *Sma*I-digested ppAsATC9a2, yielding pRGEgnDdown.

The donor plasmid pnDdownDN for deletion of 3′ region of the *niaD* gene was constructed as follows: A region within the *niaD* ORF was amplified from the genome DNA of the strain RIB40 using a primer set, 19IF-nDdownDN5F and nDdownDN5R. The downstream region of the *niaD* terminator was amplified from the genome DNA of the strain RIB40 using a primer set, nDdownDN3F and 19IF-nDdownDN3R. These two DNA fragments were fused using a primer set, 19IF-nDdownDN5F and 19IF-

nDdownDN3R, and the fused fragment was ligated with *Sma*I-digested pUC19, yielding pnDdownDN.

Genome editing plasmid for double gene deletion/integration: The genome editing plasmid pRGEgwAupgnDdown for DSB at the upstream of *wA* and the downstream of *niaD* was constructed as follows: a DNA fragment containing P*U6*, sgRNA encoding sequence, and T*U6* was amplified from the plasmid pRGEgnDdown using a primer set, Bst-PU6F and Bst-TU6R, and was ligated with *Bst*1107I-digested pRGEgwAup, yielding pRGEgwAupgnDdown.

Donor plasmid for integrating *egfp*-expression cassette into the *wA* locus: The donor plasmid pwAupEGFP was constructed as follows: the *egfp* gene was amplified from pg3′E (2) using a primer set, PamyB-C-linker-F2, and AIF-cEGFPR, and was ligated with *Sma*I-digested pUtNAN, yielding pUt-C-EGFP. A DNA fragment containing P*amyB*, *egfp*, and T*amyB* was amplified from the plasmid pUt-C-EGFP using a primer set, wADN-PamyBF and wADN-TamyBR, and was ligated with *Sma*I-digested pwAupDN, yielding pwAupEGFP.

Donor plasmid for integrating *mDsred*-expression cassette into the *niaD* locus: The donor plasmid pnDdownDR was constructed as follows: the *mDsred* gene was amplified from the plasmid pg3′DRM-CF (3) using a primer set, PamyB-C-linker-F2, and TamyB-mDsRedR, and was ligated with *Sma*I-digested pUtNAN, yielding pUt-C-mDsred. A DNA fragment containing P*amyB*, *mDsred*, and T*amyB* was amplified from the plasmid pUt-C-mDsred using a primer set, nDdDN-PamyBF and nDdDN-TamyBR, and ligated with *Sma*I-digested pnDdownDN, yielding pnDdownDR.

Quantitative PCR: The copy number of the integrated donor DNA was analyzed using TB Green™ *Premix Ex Taq*™ II (Tli RNaseH Plus) (TaKaRa) and Thermal Cycler Dice Real Time System (TaKaRa).

Western blot analysis: Preparation of cell extract, SDS-PAGE, and Western blot analysis were performed as described previously (4). Lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, and 1% NP-40) was used for preparation of cell extract. Polyacrylamide gel (12%) was used for SDS-PAGE. For detecting EGFP, Living Colors A.V. monoclonal antibody (Clontech) and peroxidase-labeled anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA) as a primary antibody and secondary antibody, respectively.

REFERENCES

1. 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: 637-42.

2. Mabashi K, Kikuma T, Maruyama J, Arioka M, Kitamoto K. 2006. Development of a versatile expression plasmid construction system for *Aspergillus oryzae* and its application to visualization of mitochondria. Biosci Biotechnol Biochem 70: 1882-9.

3. Escano CS, Juvvadi PR, Jin FJ, Takahashi T, Koyama Y, Yamashita S, Maruyama J, Kitamoto K. 2009. Disruption of the *Aopex11-1* gene involved in peroxisome proliferation leads to impaired Woronin body formation in *Aspergillus oryzae*. Eukaryot Cell 8: 296-305.

4. Okabe T, Katayama T, Mo T, Mori N, Jin FJ, Fujii I, Iwashita K, Kitamoto K, Maruyama J. 2018. BiFC-based visualisation system reveals cell fusion morphology and heterokaryon incompatibility in the filamentous fungus *Aspergillus oryzae*. Sci Rep 8: 2922.

wA mutation pattern

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Indel
Wild type          TTGATTCTAGGCTGTCTAAGAGCCATAAGCCGGTGAGGGCGCAAATCCATGGTCCTTA
-7 bps             TTGATTCTAGGCTGTCTAAGAG-------CCGGTGAGGGCGCAAATCCATGGTCCTTA
-1 bps             TTGATTCTAGGCTGTCTAAGAGCCAT-AGCCGGTGAGGGCGCAAATCCATGGTCCTTA
-24 bps            TTGATTCTAGGCTGTCTAAGAGCCAT------------------------GGTCCTTA
-8 bps             TTGATTCTAGGCTGTCTAAGAG--------CGGTGAGGGCGCAAATCCATGGTCCTTA
-6 bps             TTGATTCTAGGCTGTCTAAGAGCCA------GGTGAGGGCGCAAATCCATGGTCCTTA
-9 bps             TTGATTCTAGGCTGTCTAAGAGC---------GTGAGGGCGCAAATCCATGGTCCTTA
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yA mutation pattern

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Indel
Wild type          GATGTCCCGCGCCAAATGATTCTCACTAATGGGAAGTATCCAGGGCCTGACTTGGTGTT
-10 bps            GATGTCCCGCGCCAAATGATTCT----------AAGTATCCAGGGCCTGACTTGGTGTT
-12 bps            GATGTCCCGCGCCAAATGATTCTCA-----------ATCCAGGGCCTGACTTGGTGTT
-12 bps            GATGTCCCGCGCCAAA-----------ATGGGAAGTATCCAGGGCCTGACTTGGTGTT
-13 bps            GATGTCCCGCGCCAAA------------TGGGAAGTATCCAGGGCCTGACTTGGTGTT
-11 bps            GATGTCCCGCGCCAAATG-----------TGGGAAGTATCCAGGGCCTGACTTGGTGTT
+1 bp               GATGTCCCGCGCCAAATGATTCTCACTTAATGGGAAGTATCCAGGGCCTGACTTGGTGTT
-2 bps              GATGTCCCGCGCCAAATGATTCTCA--AATGGGAAGTATCCAGGGCCTGACTTGGTGTT
Large indel        Large deletion and Insertion from AO090102000647 locus
Large indel        Large deletion and Insertion from AO090012000538 locus
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pyrG mutation pattern

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Indel
Wild type          CGGCCGAGGACTTCCCCTACGGCTCCGAGAGGGGCCTTTTGATCCTTGCGGAGATGA
-12 bps            CGGCCGAGGACTTC-----------GAGAGGGGCCTTTTGATCCTTGCGGAGATGA
-26 bps            CGGCCGAGGACTTCCCCTACGGCTC-------------------------AGATGA
+1 bp              CGGCCGAGGACTTCCCCTACGGCTCCCGAGAGGGGCCTTTTGATCCTTGCGGAGATGA
-9 bps             CGGCCGAGGACTTCCCC---------GAGAGGGGCCTTTTGATCCTTGCGGAGATGA
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FIG S1 Mutational patterns for *wA*, *yA*, and *pyrG* in the *A. oryzae* wild strain RIB40. Solid underlines, protospacer sequence; dotted underlines, PAM sequences; hyphens, deleted nucleotides; red characters, inserted nucleotides.

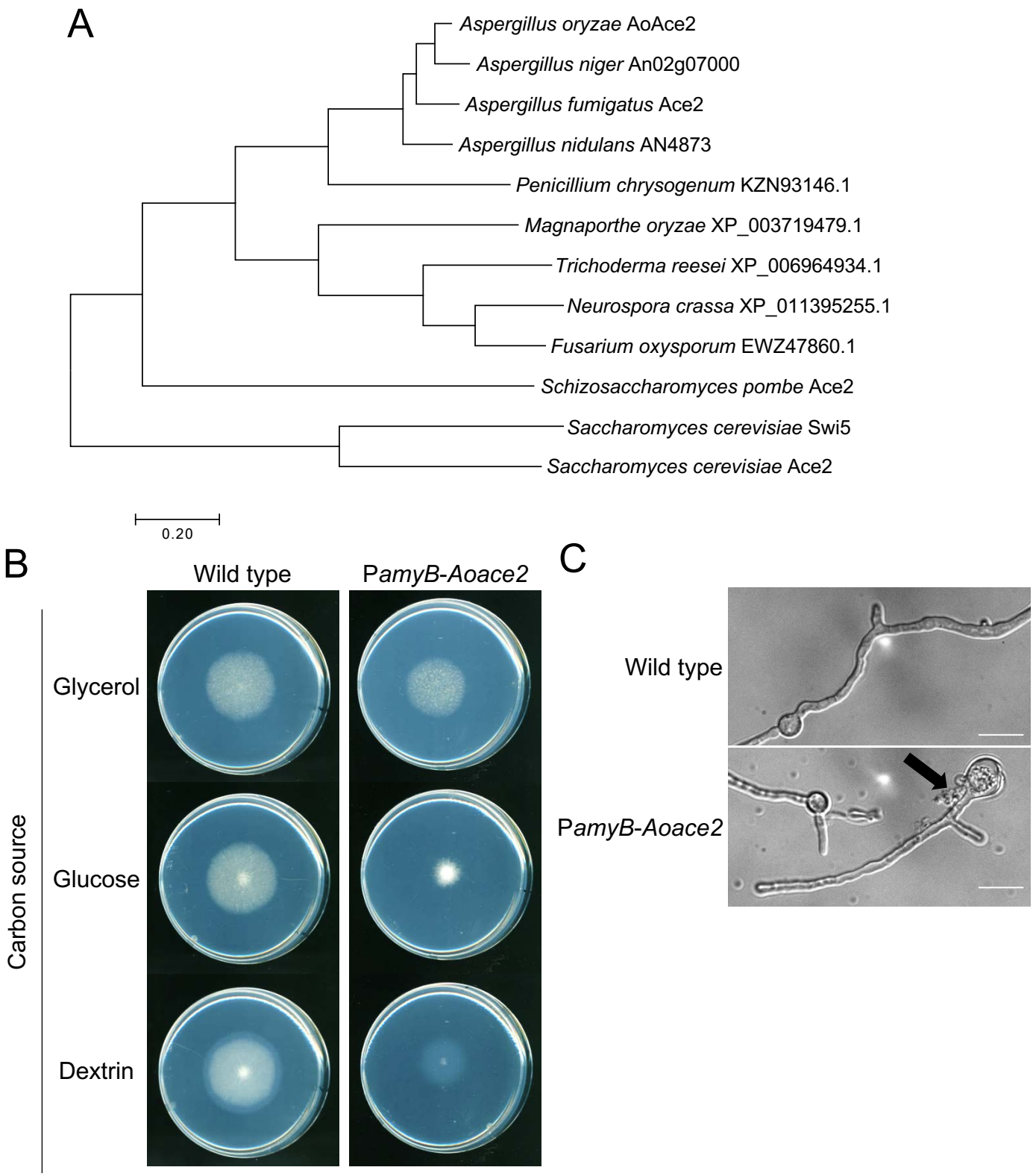

FIG S2 Growth of *A. oryzae* strain expressing *Aoace2* under the control of P*amyB*. (A) Phylogenetic tree of the AoAce2 orthologues. (B) Conidial suspension (10^4 conidia in 5 μL) of each strain was spotted onto the CD agar medium containing the indicated carbon source and 0.15% methionine, and incubated for 4 d at 30°C. Although halo was observed on the CD medium containing dextrin, the strain expressing *Aoace2* hardly grew on the medium. (C) Conidia (10^5) were inoculated in the CD medium containing dextrin as sole carbon source and 0.15% methionine, and incubated for 20 h at 30°C. Arrow indicates cell lysis. Bars: 5 μm.

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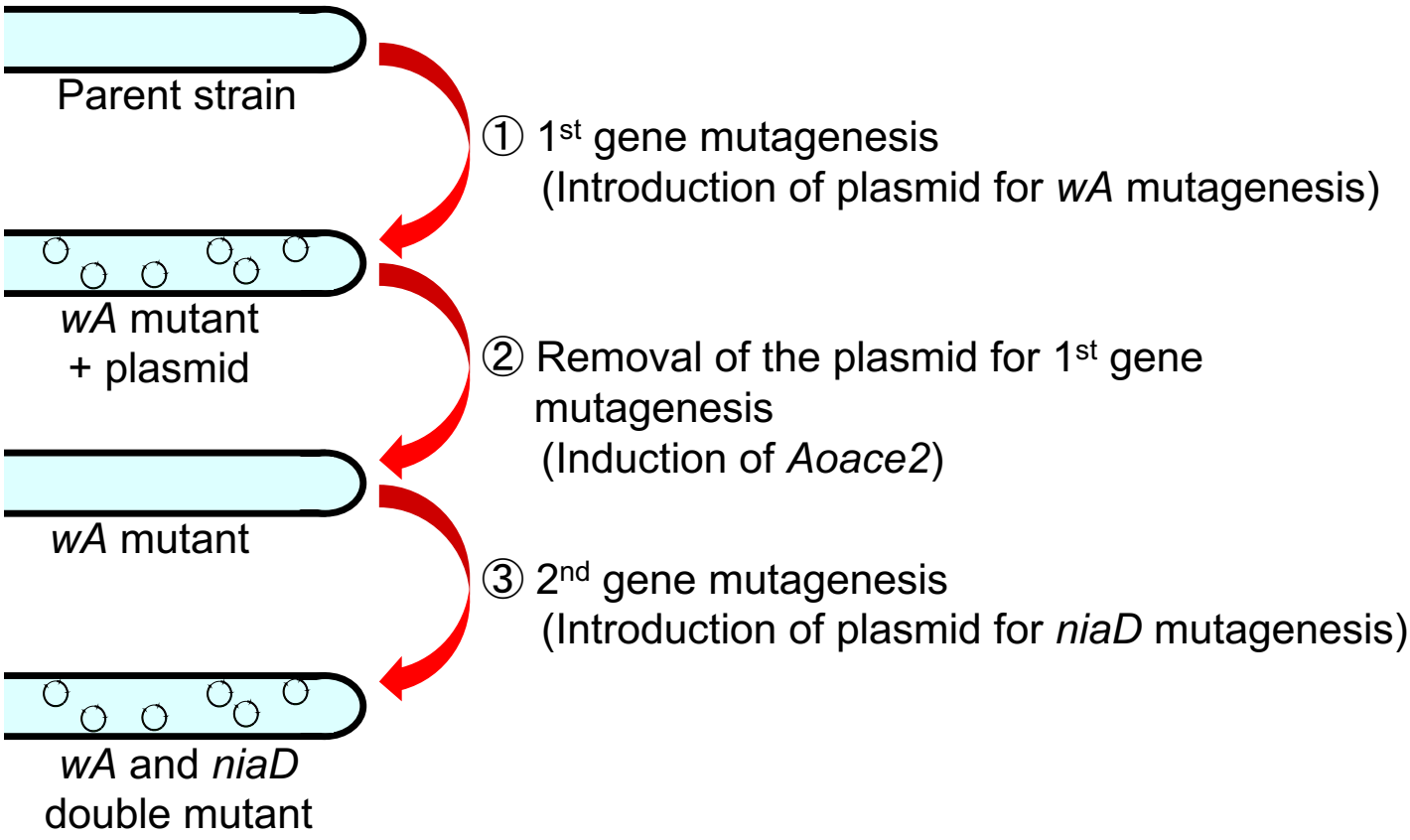

FIG S3 Strategy for the generation of the *wA niaD* double-mutant by genome-editing plasmid recycling. The genome-editing plasmid targeting *wA* is first introduced, for *wA* mutagenesis, under *Aoace2*-repressed condition. Then, the plasmid is removed from the *wA* mutant by inducing *Aoace2*. Finally, the genome-editing plasmid targeting *niaD* is introduced, for *niaD* mutagenesis, resulting in the *wA niaD* double-mutant.

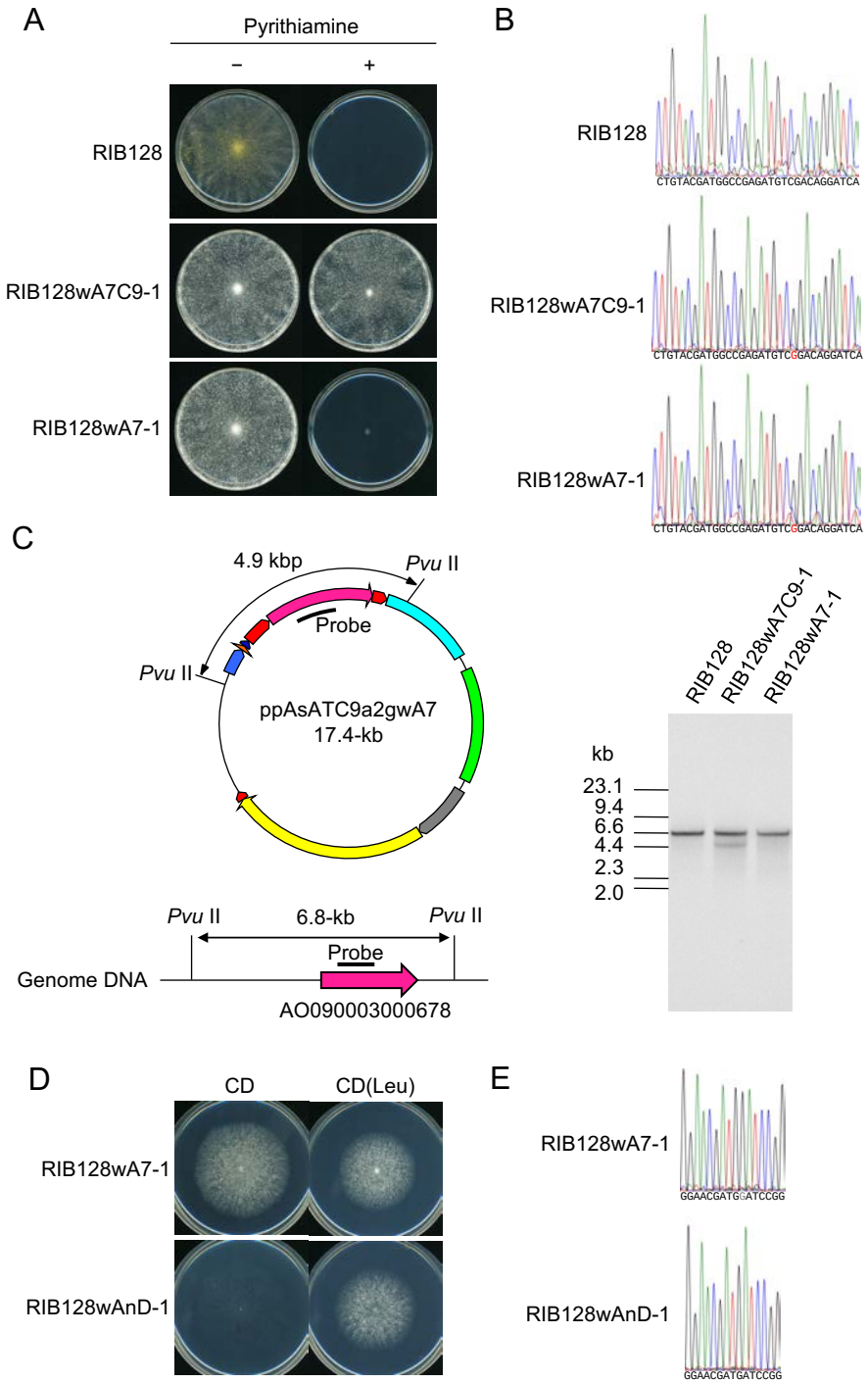

FIG S4 Generation of the *wA niaD* double-mutant of the *A. oryzae* industrial strain RIB128 by recycling of the genome editing-plasmid. (A) Pyrithiamine sensitivity test of transformants before and after plasmid removal. Conidial suspension (10^4 conidia in 5 μL) of each strain was spotted onto the CD agar medium with (+) or without (−) pyrithiamine, and incubated for 5 d at 30°C. (B) Nucleotide sequence analysis of the target sequence within the *wA* gene in the indicated strains. Red letters indicate the inserted nucleotides in the *wA* mutants. (C) Southern blot analysis to detect the removal of the genome-editing plasmid. The indicated probe detected 6.8-kb fragment of genomic DNA in the three indicated strains, and 4.9-kb fragment from the genome-editing plasmid in strain RIB128wA7C9-1. (D) Inability to assimilate nitrate by the *wA niaD* double-mutant. Conidial suspension (10^4 conidia in 5 μL) of each strain was spotted onto the indicated agar medium and incubated for 5 d at 30°C. (E) Nucleotide sequence analysis of the target sequence within the *niaD* gene in the indicated strains. Gray letter indicates the deleted nucleotide in the *niaD* mutant.

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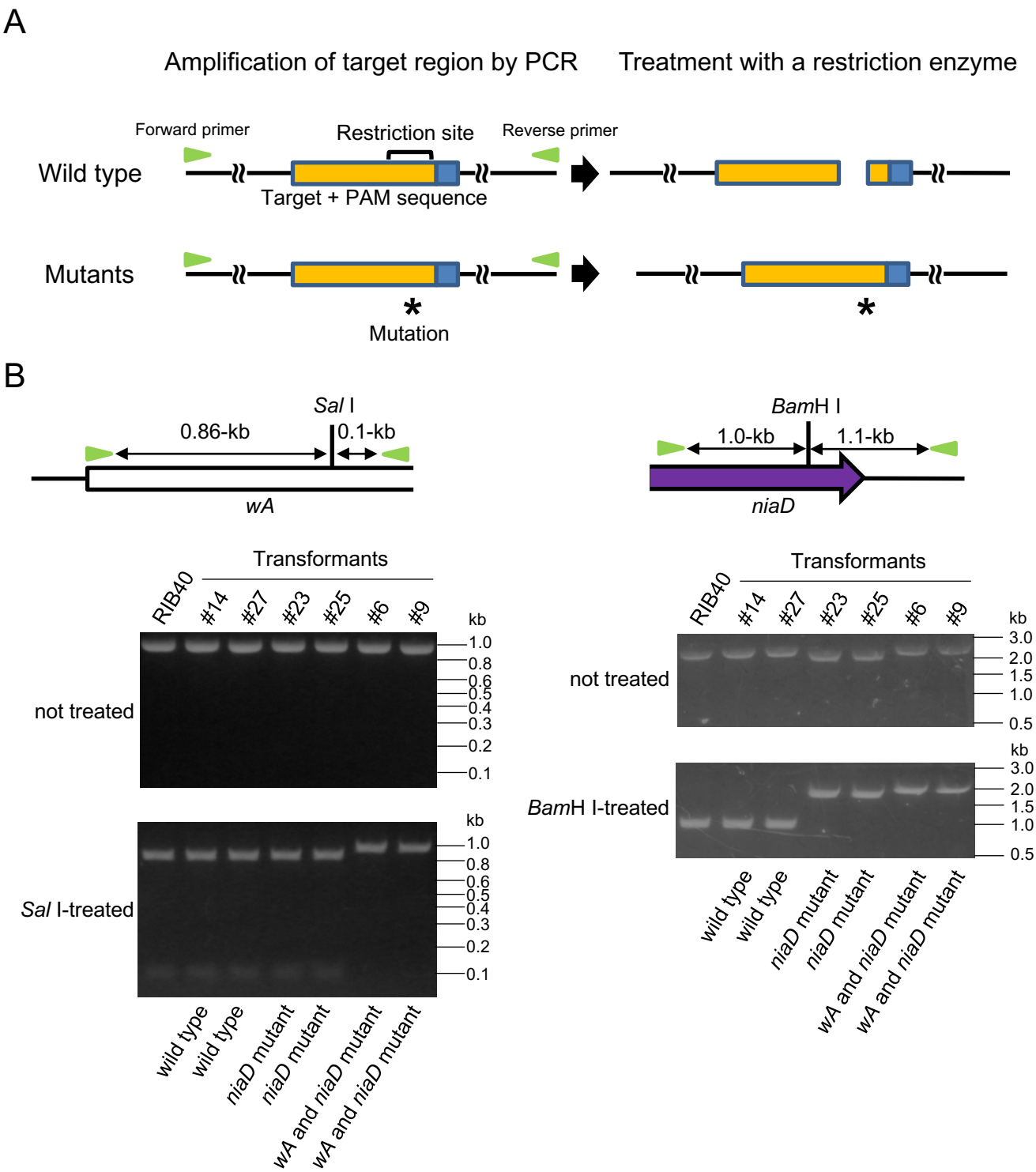

FIG S5 PCR analysis of genomic DNA of transformants generated by double mutagenesis of *wA* and *niaD* (A) Strategy for confirming the mutations by digesting the PCR products from genomic DNA using a restriction enzyme. PCR-amplified products of the wild-type strain, but not those of mutants, are digested using the restriction enzyme. (B) PCR analysis of genomic DNA and digestion analysis of the amplified products using the indicated restriction enzymes.

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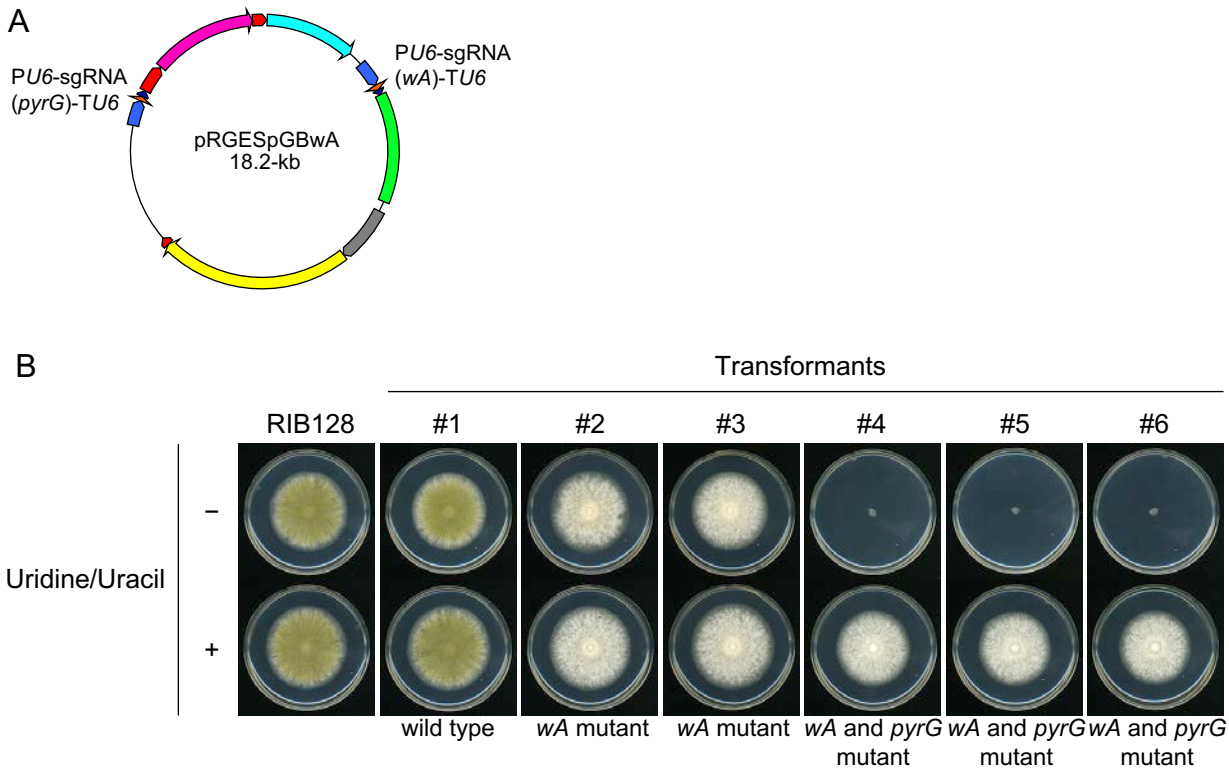

FIG S6 Double mutagenesis of *wA* and *pyrG* in the *A. oryzae* industrial strain RIB128. (A) Plasmid map of pRGESpGBwA for double mutagenesis of *wA* and *niaD*. (B) Conidial suspension (10^4 conidia in 5 μL) of each strain was spotted onto the PD agar medium with (+) or without (−) uridine/uracil, and incubated for 4 d at 30°C.

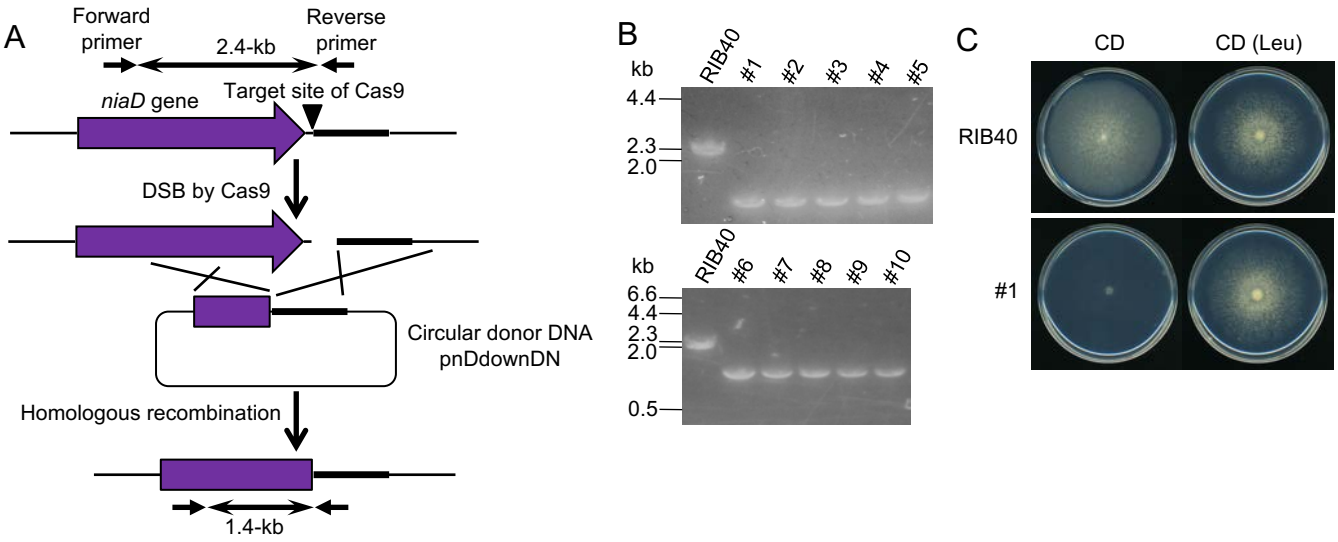

FIG S7 Deletion of *niaD* by co-transformation with the genome-editing plasmid and circular donor DNA. (A) Strategy for deletion of the 3′-region of the *niaD* gene using the CRISPR/Cas9 system. (B) PCR analysis of genomic DNA after partial deletion of *niaD*. (C) Phenotype of transformants resulting from partial deletion of *niaD*. Conidial suspension (10^4 conidia in 5 μL) of each strain was spotted onto the indicated medium and incubated for 5 d at 30°C.

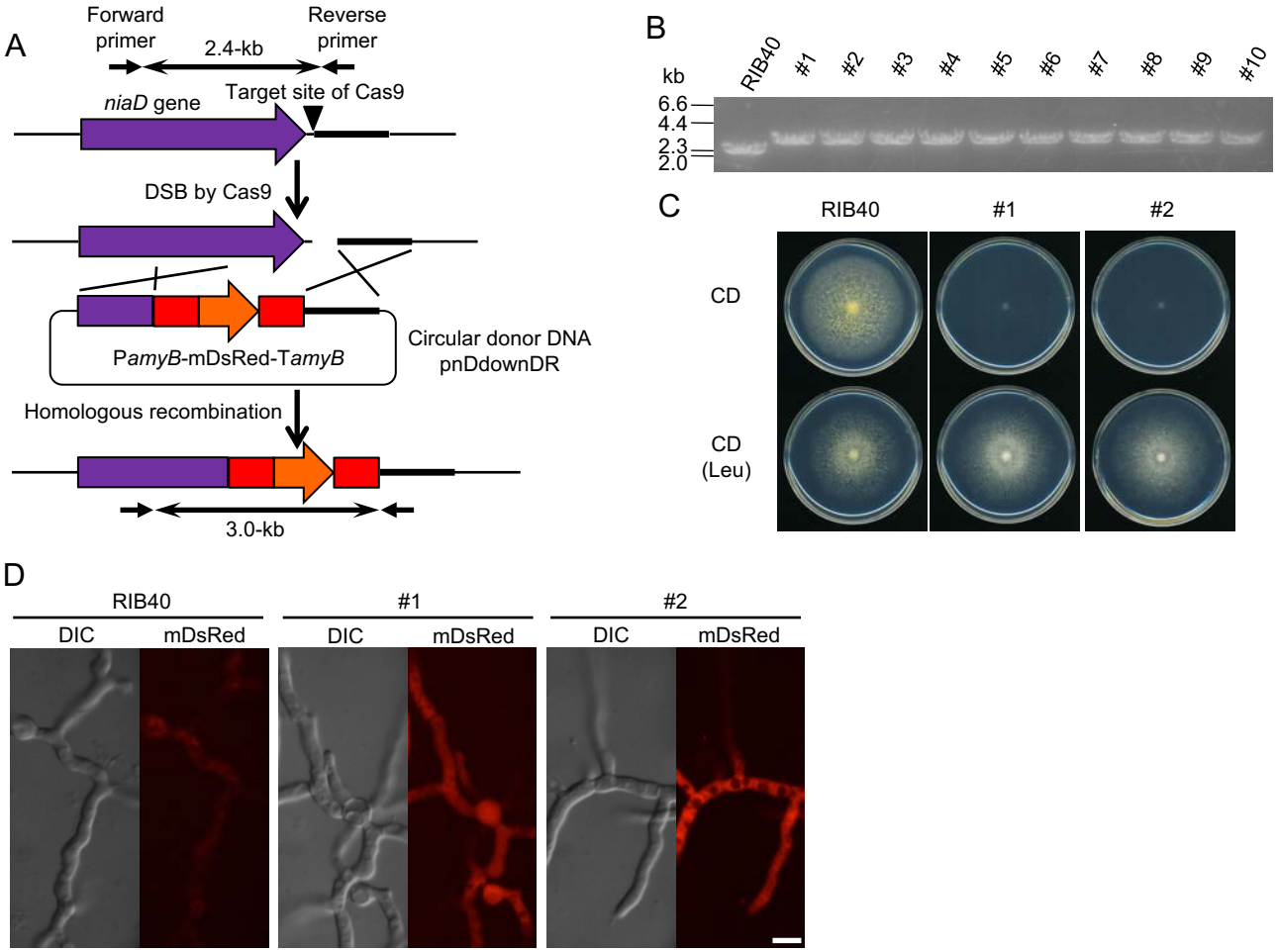

FIG S8 Integration of the mDsRed-expression cassette into the *niaD* locus by co-transformation with the genome-editing plasmid and circular donor DNA. (A) Strategy for integrating the mDsRed-expression cassette into the *wA* locus using the CRISPR/Cas9 system. (B) PCR analysis of genomic DNA after the integration. DNA fragments PCR-amplified using the primers indicated in panel A were analyzed by electrophoresis. (C) Phenotype of transformants resulting from the integration. Conidial suspension (10^4 conidia in 5 μL) of each strain was spotted onto the indicated medium and incubated for 5 d at 30°C. (D) Fluorescent microscopic analysis of transformants expressing mDsRed. Bar: 10 μm.

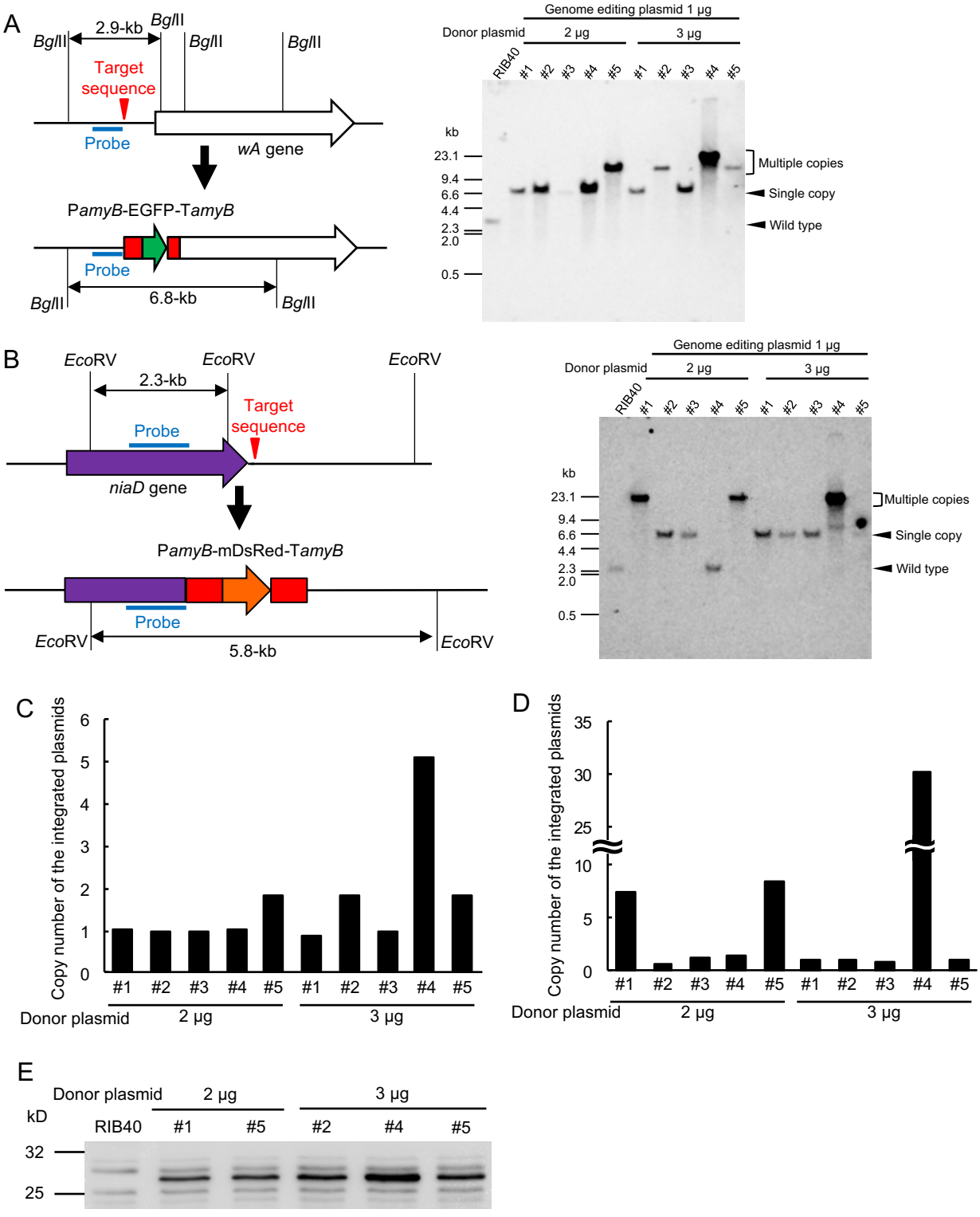

FIG S9 Investigation of the copy number of the integrated donor plasmid. (A, B) Southern blot analysis to confirm the integration of the EGFP-expression cassette into the *wA* locus (A) and the mDsRed-expression cassette into the *niaD* locus (B). As *Bgl*II and *Eco*RV do not digest each donor plasmid, the longer fragments than that indicating single integration indicate multiple integration into the target loci. (C, D) Investigation of the copy number of the integrated donor plasmids including the EGFP-expression cassette (C) and the mDsRed-expression cassette (D) by quantitative PCR. The primers used for quantitative PCR were designed within the DNA sequences indicated as probes in panels A and B. (E) Investigation of production levels of EGFP by Western blot analysis.

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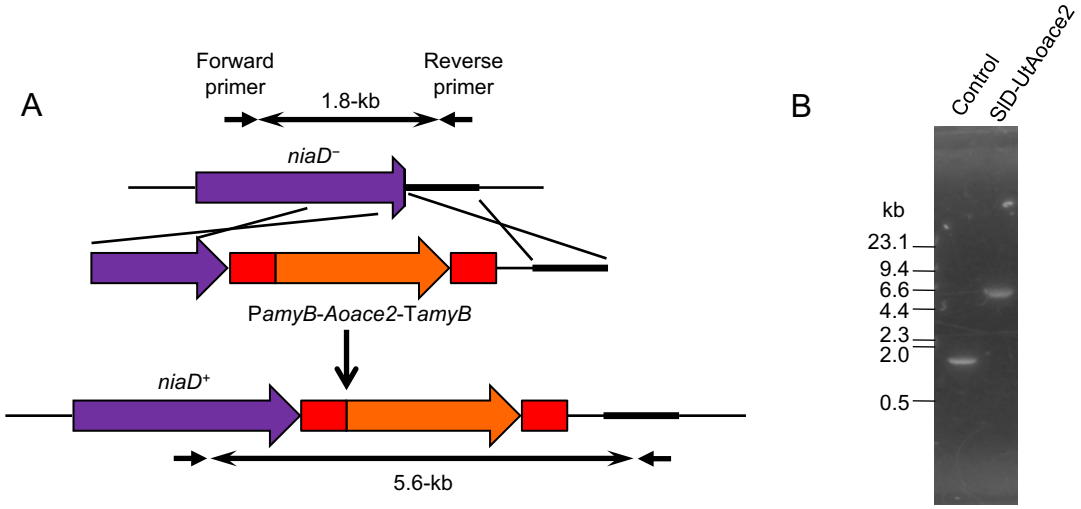

FIG S10 Generation of strains expressing *Aoace2* under the control of P*amyB* downstream of *niaD*. (A) Strategy for the construction of SID-UtAoace2. Arrows indicate the positions of primers used for PCR analysis of genomic DNA. (B) PCR analysis of genomic DNA of strain SID-UtAoace2. Strain NSID1 was used as the control.