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Gene manipulation in the Mucorales fungus *Rhizopus oryzae* using TALENs with exonuclease overexpression

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One sentence summary: Gene disruption was achieved in *Rhizopus oryzae* using Platinum TALEN (transcription activator-like effector nuclease) with newly identified exonuclease that stimulated microhomology-mediated end joining.
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Abstract

The Mucorales fungal genus *Rhizopus* is used for the industrial production of organic acids, enzymes and fermented foods. The metabolic engineering efficiency of *Rhizopus* could be improved using gene manipulation; however, exogenous DNA rarely integrates into the host genome. Consequently, a genetic tool for Mucorales fungi needs to be developed. Recently, programmable nucleases that generate DNA double-strand breaks (DSBs) at specific genomic loci have been used for genome editing in various organisms. In this study, we examined gene disruption in *Rhizopus oryzae* using transcription activator-like effector nucleases (TALENs), with and without exonuclease overexpression. TALENs with an overexpressing exonuclease induced DSBs, followed by target site deletions. Although DSBs are repaired mainly by nonhomologous end joining in most organisms, our results suggested that in *R. oryzae* microhomology-mediated end joining was the major DSB repair system. Our gene manipulation method using TALENs coupled with exonuclease overexpression contributes to basic scientific knowledge and the metabolic engineering of *Rhizopus*.

Keywords: TALEN, genome editing, Mucorales fungi, *Rhizopus oryzae*, exonuclease, metabolic engineering

Introduction

The filamentous fungi of the genus *Rhizopus* are important microorganisms that have positive applications but negative health effects on humans. Some *Rhizopus* strains, such as *Rhizopus oryzae*, are used in the industrial production of organic acids, enzymes and fermented foods (Nahas 1988, Hachmeister and Fung 1993, Zhang *et al.* 2007). However, *R. oryzae* is also the main causative microorganism of mucormycosis, which results in a severe infection with a high mortality rate (Ibrahim *et al.* 2012). Additionally, the plant pathogen *Rhizopus microsporus* causes a severe crop disease (Partida-Martinez and Hertweck 2005). Therefore, the genetic modification of *Rhizopus* increases metabolite production rates and provides significant information on pathogenic mechanism-associated gene functions.

Currently, there are several genetic tools applicable to *Rhizopus* species, including *Rhizopus niveus* (Yanai *et al.* 1990, Liou *et al.* 1992, Takaya *et al.* 1994), *Rhizopus delemar* (Horiuchi *et al.* 1995) and *R. oryzae* (Skory 2002, Michielse *et al.* 2004). For example, vectors that complement the auxotrophy of some mutants have been developed to screen transformants. Additionally, an *Agrobacterium*-mediated transformation system has been developed for efficient DNA integration into *R. oryzae*. However, the only example of targeted gene disruption in *Rhizopus* strains facilitated by spontaneous homologous recombination (HR) is that of the high-affinity iron permease gene (*ftr1*) in *R. oryzae* (Ibrahim *et al.* 2010). Targeted gene manipulation by HR is a useful strategy for gene replacement, including the use of constructs that result in gene disruption and modification. However, spontaneous HR rarely occurs in most filamentous fungi (Weld *et al.* 2006). Moreover, exogenous DNA rarely integrates into the host genomes of Mucorales (e.g. *Rhizopus*, *Mucor*, *Absidia*, *Phycomyces* and *Rhizomucor*) compared with other filamentous fungi, because their exogenous DNA connects extrachromosomally (Skory 2004). In addition, the autonomous replication of exogenous DNA may occur without an origin of replication (Revuelta and Jayaram 1986, Yanai *et al.* 1990, Benito *et al.* 1995, Appel *et al.* 2004, Skory 2004). Therefore, targeted gene manipulation by spontaneous HR using exogenous donor DNA is very rare and unreliable in Mucorales.

Recently, programmable nucleases have enabled the introduction of DNA double-strand breaks (DSBs) at specific genomic loci. Programmable nucleases are customizable site-specific nucleases that trigger genetic engineering (Sakuma and Woltjen 2014). A variety of programmable nuclease-mediated genome manipulations, such as gene knockout, gene knock-in and chromosomal rearrangement, utilizing endogenous DSB repair pathways have been performed in various organisms. Among such genetic modifications, gene disruptions resulting from short insertions and deletions that are introduced by mutagenic end-joining re-

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pair pathways, such as nonhomologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ), are particularly easy to apply. The reason for the ease of application is that these repair mechanisms are independent of HR and do not require the targeted integration of exogenous donor DNA (Kim and Kim 2014).

Transcription activator-like effector (TALE) nuclease (TALEN) is a programmable nuclease that consists of a DNA-binding domain and a DNA cleavage domain. The DNA-binding and cleavage domains originated from TALE and FokI endonucleases, respectively (Joung and Sander 2013). The efficiency of TALEN-mediated genome editing may be enhanced by several improvements. First, TALENs with variable repeats, known as Platinum TALENs, have higher DNA cleavage activity levels than conventional TALENs with constant repeats (Sakuma *et al.* 2013). Second, the introduction of TALENs along with exonuclease 1 has enhanced mutagenic efficiencies in rat fibroblasts and zygotes (Mashimo *et al.* 2013), supposedly because deletion mutations could efficiently be introduced by the resection of DSB ends by exonucleases. Another major programmable nuclease is part of the CRISPR (clustered regularly interspaced short palindromic repeats)–Cas system (Cong *et al.* 2013, Mali *et al.* 2013), which includes a Cas protein and single-guide RNA. In Mucorales, gene manipulation has been performed using the CRISPR–Cas system as the programmable nuclease (Nagy *et al.* 2017, Bruni *et al.* 2019). Although the usefulness of CRISPR–Cas system in basic research is indisputable, the rate of the off-target effects of TALENs is reportedly lower than that of the CRISPR–Cas system (Kim and Kim 2014). In addition, genome-editing efficiency of TALENs in heterochromatin target sites was reportedly higher than that of CRISPR–Cas system (Jain *et al.* 2021); therefore, gene manipulation with TALENs appears to result in safer and more versatile gene editing.

In this study, we developed a gene disruption method in *R. oryzae* using Platinum TALENs coupled with exonuclease overexpression, RoKem1 and RoXrn1, which were newly identified. We attempted to disrupt the *trpC* gene, one of the genes involved in the tryptophan biosynthesis, in which deficient mutants could be obtained by positive selection using 5-fluoroanthranilic acid (5-FAA). We found that the overexpression of one of the exonucleases, RoKem1, along with the introduction of Platinum TALENs was required for gene disruption in *R. oryzae*. This is the first report of TALEN-mediated gene disruption in Mucorales, and thus, the results contribute to our basic scientific knowledge, as well as metabolic engineering in *Rhizopus* strains.

Materials and methods

Strains, media and culture conditions

Rhizopus oryzae AM002, an adenine auxotrophic mutant, was used in this study (Fig. S1, Supporting Information). The strain was generated from *R. oryzae* NBRC5384 (NBRC, National Institute of Technology and Evaluation, Japan) by UV mutagenesis and is an *adeA*-deficient mutant. Three types of media were used in this study: (i) potato dextrose agar (BD Biosciences) was used as the complete medium containing adenine; (ii) minimum (M) medium without adenine was used for the selection of transformants; and (iii) M medium supplemented with cellobiose (CM) without adenine was used to induce expression from the *amyA* promoter. *Rhizopus oryzae* AM002 was grown on a plate containing potato dextrose agar or cultivation medium prepared in accordance with a previous report (Zhou *et al.* 1999) with some modifications. The M medium (20 g/L glucose, 1 g/L $(NH_4)_2SO_4$, 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 0.09 g/L $ZnSO_4{\cdot}7H_2O$ and 15 g/L agar) and CM medium (glucose was replaced with cellobiose in the M medium) were prepared with and without 0.006 g/L tryptophan. *Rhizopus oryzae* AM002 was cultured at 30°C for 7 days after plating.

Sample preparation for the genome and transcriptome analyses

The fungal sample cultured on a plate containing potato dextrose agar for 7 days was used for genome sequencing. The sample for the RNA-seq analysis was prepared as described later. The germination culture was prepared by inoculating *R. oryzae* NBRC5384 spores (at a final concentration of 10^3 spores/mL) into a 500-mL Erlenmeyer flask with baffles containing 200 mL of potato dextrose broth (BD Biosciences) at 27°C and 170 rpm for 3 days. After broth filtration, the supernatant was removed. The growth culture was prepared by inoculating half the cells into a 500-mL Erlenmeyer flask containing 100 mL of agar-free M medium supplemented with 5 g/L $CaCO_3$ at 27°C and 220 rpm for 40 h. After cell collection, the production culture was prepared by inoculating 6 g cells into a 200-mL Erlenmeyer flask containing 40 mL of agar-free M medium supplemented with 5 g/L $CaCO_3$ at 27°C and 170 rpm for 8 h.

Genome sequencing and RNA-seq analyses

The fungal hyphae were frozen in liquid nitrogen and fractured with metal corn using a Multi-Beads Shocker (Yasui Kikai) at 4°C. Subsequently, the whole genome was extracted using Dr. GenTLE (from Yeast) High Recovery (TaKaRa), and total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen), in accordance with the manufacturers' protocols. The draft genome sequencing using the HiSeq 2000 System (Illumina) was performed by Hokkaido System Science. The mRNA sequencing libraries were prepared using Illumina TruSeq RNA Sample Prep Kit v2 (Illumina) and sequenced using the MiSeq System (Illumina) with MiSeq Reagent Kit (300 cycles, Illumina). Sequencing data produced by the MiSeq System were analyzed using CLC Genomics Workbench (CLC bio).

Development of *R. oryzae* expression plasmids

For exogenous gene expression in *R. oryzae*, the following two vectors were constructed: pAmy_L_empty and pAmy_R_empty, which contained an empty gene cassette harboring the *amyA* promoter and *pdcA* terminator, with and without the *adeA* selection marker, respectively. The primers used and isolated promoter sequences are listed in Table S1 and Supplementary Sequences (Supporting Information), respectively.

pAde1, the *adeA*-expressing plasmid, was constructed by fusing the following two fragments using an In-Fusion HD Cloning Kit (TaKaRa): (i) the vector fragment from pPTR I DNA (TaKaRa) amplified with pPTR1-ptrProup-F and pPTR1-Nae1up-R primers and (ii) the insert *adeA*-gene fragment from the *R. oryzae* genome DNA amplified with ade1-up6 and ade1-down6 primers.

The pAmy_L_empty was constructed by fusing the following three fragments using an In-Fusion HD Cloning Kit: (i) the vector fragment from pAde1 amplified with pPTR1-Pst1-F and pPTR1-Pst1-R primers; (ii) the insert *amyA*-promoter fragment from the *R. oryzae* genome amplified with pPTR1-amyApro-F and amyApro-pdcAter-R primers; and (iii) the insert *pdcA*-terminator fragment from the *R. oryzae* genome amplified with amyApro-pdcAter-F and pdcAter-pPTR1-R.

The pAmy_R_empty was constructed by fusing the following three fragments using an In-Fusion HD Cloning kit: (i) the vec-

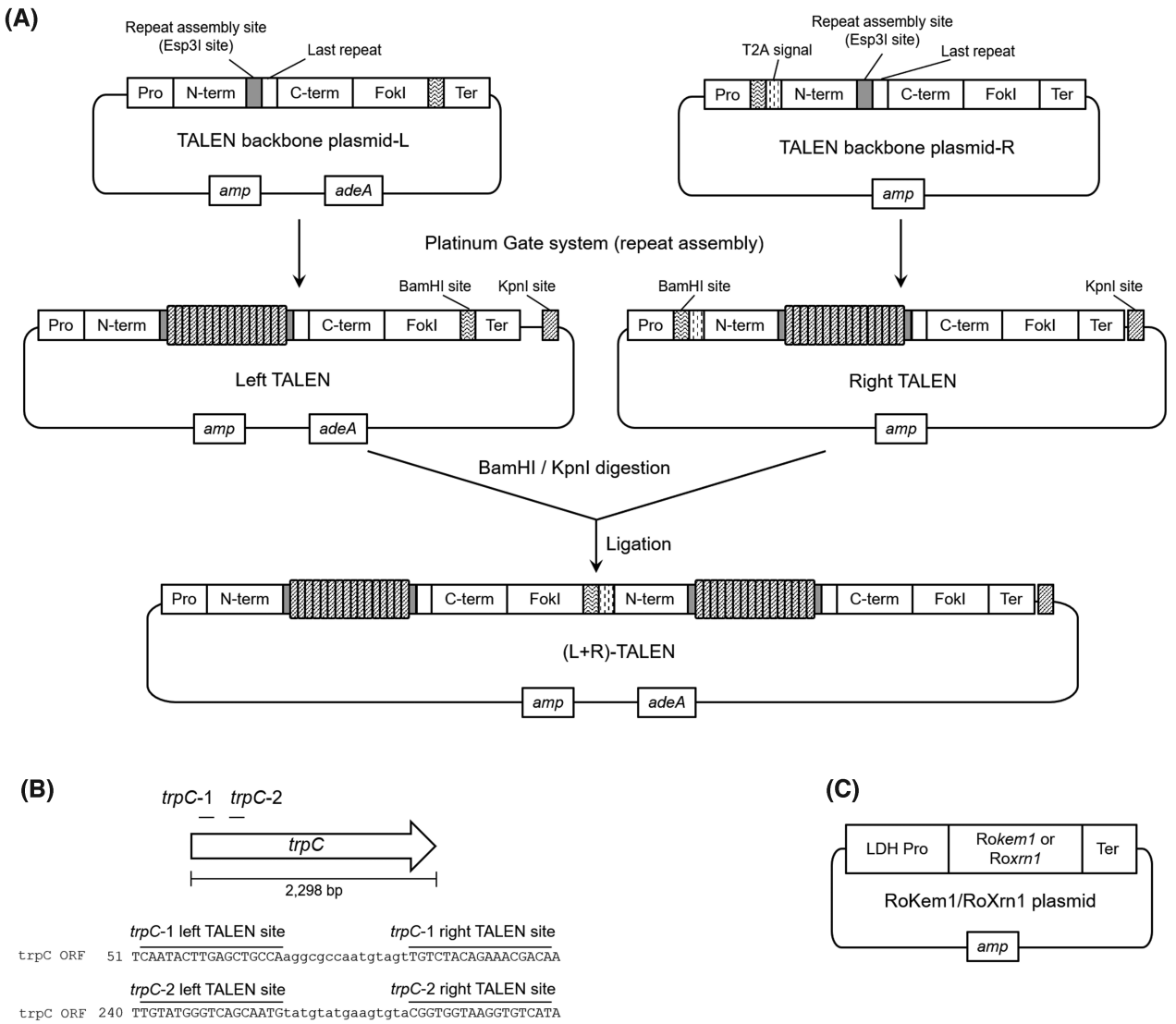

Figure 1. Schematic illustrations of plasmids and TALEN target sites. **(A)** The constructed TALEN system in *R. oryzae* with Platinum Gate system; **(B)** *trpC* gene and TALEN target sites; and **(C)** exonuclease expression plasmids. Pro, promoter; N-term, N-terminal domain of TALE; C-term, C-terminal domain of TALE; Ter, terminator; amp, ampicillin resistance gene. *trpC*-1 and *trpC*-2 indicate TALEN target sites.

tor fragment from pUC18 amplified with pPTR1-sal1-F and pPTR1-sal1-R primers; (ii) the insert *amyA*-promoter fragment from the *R. oryzae* genome amplified with sal1-amyApro-F and amyApro-pdcAter-R primers; and (iii) the insert *pdcA*-terminator fragment from the *R. oryzae* genome amplified with amyApro-pdcAter-F and pdcAter-sal1-R primers.

After the cloning, BamHI and Esp3I sites in the *adeA* gene and *pdcA* terminator, respectively, were removed from pAmy_L_empty. Two and one Esp3I sites in the pUC18 vector and *pdcA* terminator, respectively, were removed from pAmy_R_empty.

Construction of TALEN destination vectors for *R. oryzae*

To construct the TALEN destination vectors for *R. oryzae*, we cloned the Platinum TALEN scaffold into *R. oryzae* expression vectors, with coding sequence modifications based on the previously established fast unification of separate endonucleases (FUSE) system (Tokumasu *et al.* 2014). Briefly, modified Platinum TALEN scaffolds for the Golden Gate assembly containing an NN or NI module as the last repeat were cloned into the pAmy_L_empty and pAmy_R_empty vectors to create pAmy_L and pAmy_R, respectively. For the left TALEN, a BamHI site was added just upstream of the stop codon. For the right TALEN, a T2A-coding sequence (Daniels *et al.* 2014) containing a BamHI site was added just downstream of the start codon (Fig. 1A). pLdh_L and pLdh_R, as well as pAdh_L and pAdh_R, were constructed by replacing the *amyA* promoter in pAmy_L and pAmy_R with the *ldhA* and *adh* promoters, respectively.

TALEN target site design, DNA-binding repeat assembly and TALEN cDNA unification using the FUSE method

Two TALEN target sites in the *trpC* locus, *trpC*-1 and *trpC*-2, were designed using the TALEN Targeter web tool (Doyle *et al.* 2012) (https://tale-nt.cac.cornell.edu/node/add/talen). The important residues involved in the activity of TrpC in *Escherichia coli* are K114, E163 and N184 (Kos *et al.* 1988). According to a homology search, these residues were equivalent to K356, E405 and N426, respectively, of TrpC in *R. oryzae*. The TALEN target sites were designed upstream of K356. The left and right target sequences of

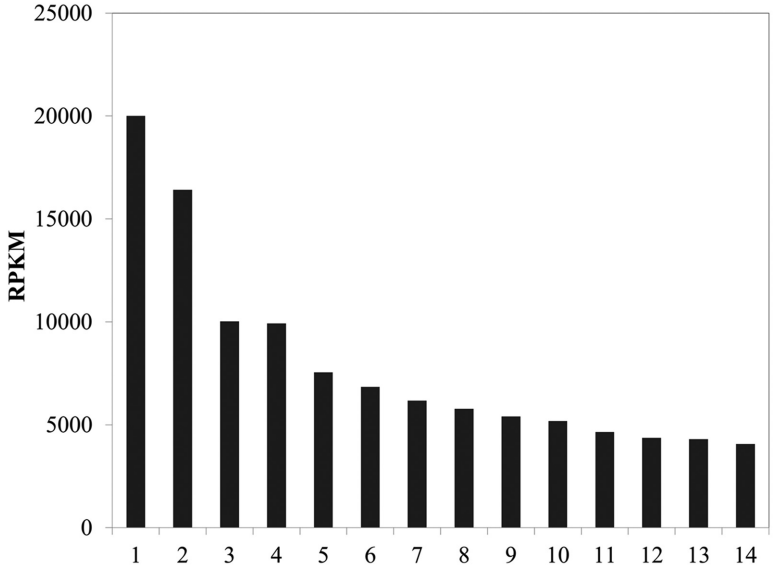

Figure 2. Identification of highly expressed *R. oryzae* genes by RNA-seq analysis. Reads per kilobase of exon per million mapped reads (RPKM) of the top 14 highly expressed genes are shown. 1, unknown; 2, L-lactate dehydrogenase (*ldhA*); 3, glyceraldehyde-3-phosphate dehydrogenase; 4, alcohol dehydrogenase (*adh*); 5, glucoamylase; 6, elongation factor 1-alpha; 7, enolase; 8, bZIP transcription factor; 9, concanamycin-induced protein C (*cipC*); 10, fructose-bisphosphate aldolase; 11, LysM domain-containing protein; 12, NADH-ubiquinone oxidoreductase MLRQ subunit; 13, calcium-dependent lipid-binding domain-containing protein; 14, pyruvate decarboxylase (*pdcA*).

trpC-1 were 5′-caatacttgagctgcca-3′ and 5′-tgtcgtttctgtagaca-3′, respectively, and those of *trpC*-2 were 5′-tgtatgggtcagcaatg-3′ and 5′-atgacaccttaccaccg-3′, respectively (Fig. 1B). The DNA-binding repeats of the TALENs were assembled as previously described (Sakuma *et al.* 2013). Subsequently, to obtain the all-in-one TALEN vector, TALEN-T2A-TALEN, the left and right TALEN expression cassettes were unified by restriction digestion with BamHI and KpnI, followed by ligation (Fig. 1A).

Cloning of *R. oryzae* exonucleases

The coding sequences of two candidate *R. oryzae* exonuclease genes, Ro*kem1* (Accession No. LC638492) and Ro*xrn1* (Accession No. LC638493), were amplified using the *R. oryzae* NBRC5384 genome as a template with the primer sets ldhApro-en1-F/en1-pdcAter-R and ldhApro-en2-F/en2-pdcAter-R, respectively. The vector fragment was amplified with the primers pdcAter-F and ldhApro-R, using pLdh_R as a template. The Ro*kem1* and Ro*xrn1* gene fragments were cloned independently into the vector fragment using an In-Fusion HD Cloning Kit (Fig. 1C).

Transformation

The transformation of *R. oryzae* AM002 was performed using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories) as previously described (Skory 2002) with slight modifications. To linearize the plasmids, the pAmy-, pAdh- and pLdh-based TALEN vectors were digested with ScaI, ScaI and NdeI, respectively. pLdh-RoKem1 and pLdh-RoXrn1 were digested with PstI and SphI, respectively. The linearized plasmid weight ratios for the transformation were as follows: for the samples without exonuclease, the left TALEN to right TALEN ratio was ∼1:3, whereas for the samples with exonuclease, the left TALEN:right TALEN:exonuclease ratio was ∼1:3:3 and the (L + R)-TALEN to exonuclease ratio was ∼1:4. The amount of total DNA was 1–2 μg per bombardment. The preparation of DNA-coated tungsten particles was performed as previously described (Herzog *et al.* 1996) with slight modifications. Briefly, tungsten particles were coated with the following mixture: 12.5 μL of tungsten particle solution, 1 μL DNA solution (1–2 mg/mL), 12.5 μL of 2 M $CaCl_2$ and 5 μL of 0.1 M spermidine. Spores of AM002 on filter paper were transformed.

Screening of 5-FAA-resistant mutants and tryptophan auxotroph identification

Spores transformed with TALEN plasmids were grown on CM or M medium (CM for pAmy and M for pAdh and pLdh) supplemented with tryptophan at 30°C for 7 days. After sporulation-stimulated segregation, mutant spores were screened in 5-FAA medium (10 g/L glucose, 3.36 g/L yeast nitrogen without amino acids, 5 g/L 5-FAA, 0.006 g/L tryptophan and 15 g/L agar) adjusted to pH 6 with 1 N NaOH as the first screening. In the second screening, the colonies were grown again in the 5-FAA medium. Tryptophan auxotrophy among the grown colonies in the second screening was identified using M medium with or without tryptophan.

Analysis of *trpC* mutations

For genotyping, genomic PCR products amplified region around the target site were sequenced directly or after bacterial cloning into the pUC18 vector. KOD FX Neo (TOYOBO) or PrimeSTAR MAX DNA polymerase (TaKaRa) was used to amplify the insert and cloning vector. The hyphae of tryptophan auxotrophic mutants were suspended in PCR buffer [100 mM Tris–HCl (pH 9.5), 1 M KCl and 10 mM EDTA], heated at 95°C for 10 min, vortexed and used as templates for genomic PCR. The genomic DNA fragments containing the two TALEN target sites, *trpC*-1 and *trpC*-2, were amplified with trpC-up-F2/trpC-down-R2 primers and trpC-up-F4/trpC-down-R3 primer pairs, respectively. The vector fragment was amplified with the primer set trpC-trpCR3-F/pUC18-trpCF4-R. The sequences of the primers are shown in Table S1 (Supporting Information). The PCR products amplified with trpC-up-F4 and trpC-down-R3 were cloned into the vector fragment prepared as described earlier using an In-Fusion HD Cloning Kit, and then, the

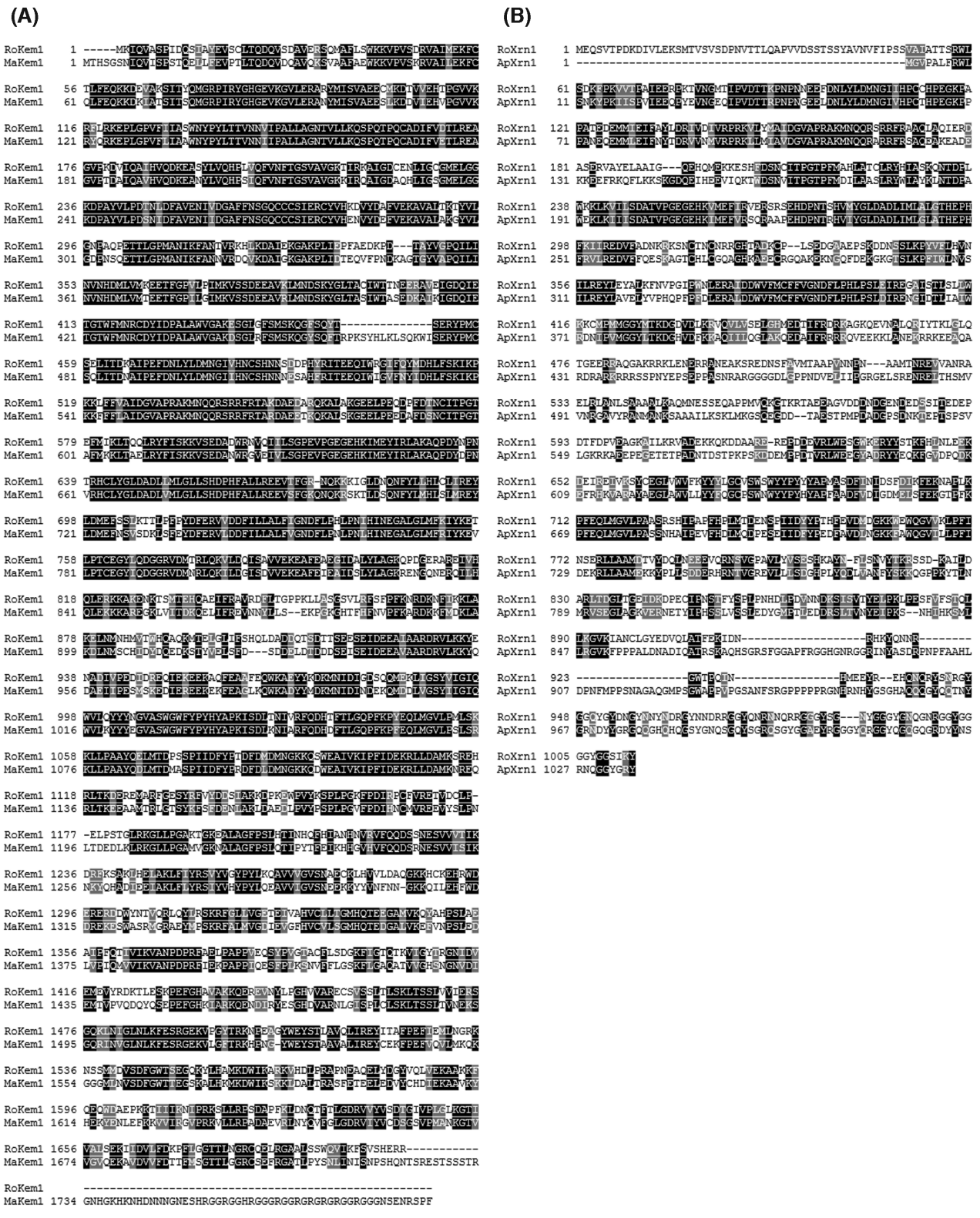

Figure 3. Amino acid alignments of potential exonuclease orthologs in *R. oryzae* and known exonucleases. Alignments of **(A)** the newly identified RoKem1 and Kem1 from *M. ambiguus* (MaKem1) and **(B)** the newly identified RoXrn1 and Xrn1-like protein from *A. parasiticus* (ApXrn1). The Xrn 5–3 exonuclease N-terminal domains of MaKem1 and ApXrn1 are located at 471–1429 and 1–869 aa, respectively.

Table 1. Evaluation of *trpC* gene disruption by TALEN with exonucleases.

Target site	Promoter	Joint or severalty	Exonuclease	First screening of 5-FAA	Second screening of 5-FAA	Tryptophan auxotrophic mutants
trpC-1	*amy*	J	RoKem1	70	47	0
		S	RoKem1	28	9	0
	adh	J	RoKem1	28	3	0
		S	RoKem1	56	14	5
	ldh	J	RoKem1	28	13	0
		S	RoKem1	21	7	0
	adh	J	RoXrn1	28	6	0
		S	RoXrn1	28	8	0
	ldh	J	RoXrn1	21	7	0
		S	RoXrn1	21	6	0
trpC-2	*amy*	J	RoKem1	76	51	0
		S	RoKem1	21	5	0
	adh	J	RoKem1	40	8	0
		S	RoKem1	21	6	0
	ldh	J	RoKem1	21	1	0
		S	RoKem1	22	1	0
	adh	J	RoXrn1	N.T.	N.T.	N.T.
		S	RoXrn1	21	7	0
	ldh	J	RoXrn1	21	5	0
		S	RoXrn1	49	28	0

Promoter, promoter for the expression of TALENs; joint or severalty, TALENs in a single vector (joint; J) or in multiple vectors (severalty; S); exonuclease, kinds of exonuclease expressed with TALENs; first screening of 5-FAA, number of colonies grown on the initial 5-FAA medium; second screening of 5-FAA, number of colonies grown on the second 5-FAA medium; tryptophan auxotrophic mutants, number of mutants that did not grow in the absence of tryptophan from colonies identified in the second 5-FAA screening; N.T., not tested.

Figure 4. Sequence analysis of the TALEN cleavage site in the tryptophan auxotrophic mutants. **(A)** TALEN target site in *trpC*-1 and positions of base deletions in the tryptophan auxotrophic mutants 1–5. Black bars indicate TALEN-binding sites. Gaps generated by deletion are shown as dashes. **(B)** DSB repair junctions in the tryptophan auxotrophic mutants 1–5. Shaded backgrounds indicate microhomologies of the 3′ and 5′ termini in each junction. Gaps generated by deletion are shown as dashes.

cloned plasmids were sequenced. The PCR products amplified with trpC-up-F2 and trpC-down-R2 were sequenced directly.

Results

Vector system for the constitutive or inducible expression of TALENs in *R. oryzae*

To evaluate promoters in *R. oryzae* NBRC5384, an RNA-seq analysis was performed using total RNA collected from a fungal sample grown on M medium. Using the read counts of the RNA-seq analysis, we identified the *ldhA* and *adh* promoters as being stronger than the *pdcA* promoter, which had been previously characterized for exogenous expression (Mertens *et al.* 2006) (Fig. 2). Additionally, the *amyA* promoter was chosen as an inducible promoter (Mertens *et al.* 2006). In our system, the left and right TALENs were expressed either using independent plasmids or a single plasmid containing an integrated coding sequence divided by the T2A peptide driven by a single promoter (Fig. 1A).

Targeted gene disruption using TALENs

To examine the gene disruption activity of TALENs in *R. oryzae*, we targeted the *trpC* gene, which produces deficient mutants that can be positively selected using 5-FAA (Toyn *et al.* 2000) (Fig. S2, Supporting Information). Two kinds of target sequences were designed at the *trpC* locus using the TALEN Targeter web tool (Fig. 1B). Both separate and unified TALEN expression plasmids for *R. oryzae* were

constructed using three promoters as described earlier. Using various transformant conditions with each TALEN pair, we obtained 5-FAA-resistant clones; however, they were not tryptophan auxotrophic mutants, because they grew in the M medium without tryptophan. These results indicated that it was difficult to disrupt genes effectively in *R. oryzae* using TALENs alone.

Identification of exonuclease orthologs in *R. oryzae*

The overexpression of exonuclease 1 increases the gene disruption efficiency of TALENs in rat zygotes (Mashimo *et al.* 2013). Consequently, we searched for exonuclease orthologs in *R. oryzae* to facilitate TALEN-mediated genome editing. This search was performed using the in-house genome sequence data. Although typical exonucleases were not annotated, multifunctional exoribonucleases that have exonuclease activities (Heyer *et al.* 1995) were annotated. RoKem1, consisting of 1704 aa, shares a 68% identity with KEM1 in *Mucor ambiguus*, and RoXrn1, consisting of 1013 aa, shares a 43% identity with XRN1-like protein in *Aspergillus parasiticus*.

Targeted gene disruption using TALENs with exonucleases

Two exonuclease orthologous genes were cloned from *R. oryzae*, and their expression vectors, pLdh-RoKem1 and pLdh-RoXrn1, were constructed (Figs 1C and 3A and B). Each pair of TALEN and exonuclease plasmids was co-transformed, and the transformants were screened using 5-FAA plates. Many 5-FAA-resistant clones were obtained, similar to the results without the exonuclease; however, here, five tryptophan auxotrophic mutants were contained (Table 1).

Sequencing analysis of *trpC*-deficient mutants

A sequencing analysis was performed at the *trpC* locus of the five tryptophan auxotrophic mutants described earlier, as well as the loci of 14 5-FAA-resistant clones that were not identified as tryptophan auxotrophic mutants. As expected, the clones without tryptophan auxotrophy did not have *trpC* mutations. However, one of the five tryptophan auxotrophic mutants (No. 1) harbored a 238-bp deletion, and the others (Nos 2–5) harbored 709-bp deletions in the upstream region starting from the TALEN cleavage site (Fig. 4A). Although the main DSB repair pathway is NHEJ in fungi, like many eukaryotic organisms (Ninomiya *et al.* 2004), these deletions are the results of MMEJ repair, because there were 2–3-bp direct repeats at both end-joining sites (Fig. 4B).

Discussion

We attempted to develop a gene disruption method in *R. oryzae* using programmable nucleases because there have been only two examples of targeted gene disruption in *Rhizopus* strains (Ibrahim *et al.* 2010, Bruni *et al.* 2019). Although genome editing technology has been applied to various fungal organisms (Arazoe *et al.* 2015, Katayama *et al.* 2016, Liu *et al.* 2017), only one successful example of genome editing using the CRISPR–Cas system in *Rhizopus* strains has been reported (Bruni *et al.* 2019). This is the first report of a TALEN-mediated gene disruption in Mucorales.

We attempted to disrupt the target gene using a template-free deletion induced by a TALEN in *R. oryzae* because exogenous DNA is rarely integrated into the host genomes of *Rhizopus* strains compared with other organisms. A gene deletion at the specific target genomic loci was not observed in *R. oryzae* using a TALEN only, even though we used the highly active Platinum TALEN. During the mutant screening process, we determined that most of the 5-FAA-resistant clones were not actual mutants. This may result from the transcriptional repression of the *trpC* gene by the temporal binding of TALEN molecules at the target locus. Therefore, we performed a three-step selection (two screenings of growth in 5-FAA and tryptophan auxotrophic selection) process.

Despite the imperfect selection procedure, we successfully obtained genome-edited clones resulting from exonuclease overexpression along with TALEN introduction. The genomic cleavage by the TALEN, followed by the exonuclease-driven resectioning, might result in relatively long deletions spanning hundreds of bases. In addition, importantly, we found traces of MMEJ repair in these deletion events, although NHEJ is generally dominant in the DSB repair of fungi.

This may be explained by the low expression levels of genes involved in DSB repair pathways other than MMEJ. In fact, the RNA-seq data suggested that the expression level of *ku70*, a major player in NHEJ repair, was very low in *R. oryzae*. The expression level of replication protein A (*rpa*) was also low. RPA is a single-strand DNA-binding protein that plays an essential role in DNA replication, recombination and repair (especially important for HR repair). Another possible reason is the low functionality of the molecules, such as RPA, which suppresses MMEJ, as indicated by the increase in MMEJ activity in RPA mutants compared with wild-type strains (Deng *et al.* 2014, McVey 2014). Although the *RPA* gene in *R. oryzae* has a conserved D228, which, if mutated, increases the frequency of MMEJ (Deng *et al.* 2014), an ∼200-aa C-terminal part of RPA was lost in *R. oryzae* in comparison with other organisms (Fig. S3, Supporting Information). Further investigation is needed to clarify the detailed mechanisms of the DSB repair pathway in *R. oryzae*. For genetic engineering, the gene insertion strategy based on MMEJ, called PITCh (Nakade *et al.* 2014), will help advance gene manipulations in *R. oryzae*.

To our best knowledge, this study is the first report of the employment of TALEN/exonuclease approach in fungal strains; however, we believe that our approach can be applied in a wide range of fungal strains because our approach is not affected by the HR efficiency of hosts, which is a major limitation to apply genome editing in various organisms. In addition, TALEN activity in heterochromatin target sites is reportedly higher than the activity of CRISPR–Cas system (Jain *et al.* 2021); therefore, the combination with TALENs and exonuclease might be a broadly accessible strategy of genome editing even in a silenced gene locus. Since the mutants created by genome editing can stably be maintained, our approach will contribute to the implementation of the collection of fungal mutant resources.

In summary, our study demonstrated that genome editing using TALENs with exonuclease overexpression was applicable in *R. oryzae*. Our method contributes to basic scientific knowledge and will aid in the metabolic engineering of *Rhizopus* strains.

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Supplementary data

Supplementary data are available at *FEMSLE* online.

Conflict of interest. None declared.

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