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Detailed analysis of targeted gene mutations caused by the Platinum-Fungal TALENs in *Aspergillus oryzae* RIB40 strain and a *ligD* disruptant

Osamu Mizutani,[1,*] Takayuki Arazoe,[2] Kenji Toshida,[1] Risa Hayashi,[1] Shuichi Ohsato,[2] Tetsushi Sakuma,[3] Takashi Yamamoto,[3] Shigeru Kuwata,[2] and Osamu Yamada[1]

National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan,[1] Graduate School of Agriculture, Meiji University, 1-1-1 Higashi-Mita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan,[2] and Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan[3]

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Transcription activator-like effector nucleases (TALENs), which can generate DNA double-strand breaks at specific sites in the desired genome locus, have been used in many organisms as a tool for genome editing. In Aspergilli, including *Aspergillus oryzae*, however, the use of TALENs has not been validated. In this study, we performed genome editing of *A. oryzae* wild-type strain via error of nonhomologous end-joining (NHEJ) repair by transient expression of high-efficiency Platinum-Fungal TALENs (PtFg TALENs). Targeted mutations were observed as various mutation patterns. In particular, approximately half of the PtFg TALEN-mediated deletion mutants had deletions larger than 1 kb in the TALEN-targeting region. We also conducted PtFg TALEN-based genome editing in *A. oryzae ligD* disruptant (*ΔligD*) lacking the *ligD* gene involved in the final step of the NHEJ repair and found that mutations were still obtained as well as wild-type. In this case, the ratio of the large deletions reduced compared to PtFg TALEN-based genome editing in the wild-type. In conclusion, we demonstrate that PtFg TALENs are sufficiently functional to cause genome editing via error of NHEJ in *A. oryzae*. In addition, we reveal that genome editing using TALENs in *A. oryzae* tends to cause large deletions at the target region, which were partly suppressed by deletion of *ligD*.

[**Key words:** *Aspergillus oryzae*; Genome editing; Transcription activator-like effector nucleases; *ligD*; Large deletion; Nonhomologous end-joining]

Transcription activator-like effector nucleases (TALENs) are fusion proteins of a TAL effector domain, homologous to the DNA-binding domain secreted by *Xanthomonas* bacteria, and a non-specific DNA cleavage domain, homologous to the catalytic domain of the FokI endonuclease (1). The TAL effector domain contains tandem repeats of a highly conserved 34 amino acid domain. Within each domain are variable 12th and 13th amino acids that together constitute the repeat variable diresidue (RVD). Each repeated domain recognizes a single nucleotide, and the specificity of nucleotide recognition depends on RVD. The four different RVDs, HD, NG, NI, and NN, correspond to C, T, A, and G, respectively (2). The FokI domain must dimerize to form an active nuclease, so two TALENs are required to cleave a specific DNA region. TALENs have been used to modify the genome via DNA double-strand break (DSB) in many organisms (2,3).

Recently, Sakuma et al. (4) reported that TALENs harboring non-RVD variations (at the 4th and 32nd residues), known as Platinum TALENs, have higher activity than conventional TALENs, provided that the Platinum TALENs are efficiently constructed using the Platinum Gate system. In addition, Platinum-Fungal TALEN (PtFg TALEN), which was applied with the Platinum Gate TALEN system in the rice blast fungus *Pyricularia oryzae* by Arazoe et al. (5), can dramatically improve the efficiency of homologous recombination-mediated targeted gene replacement. However, the usability of the PtFg TALEN for genome editing via error-prone non-homologous end-joining (NHEJ) has not been studied.

Aspergillus oryzae is a major source of commercial enzymes used for food processing and the production of fermented foods and beverages (6–9). Although *A. oryzae* is one of the most important microorganisms in the industry, because it has no known sexual cycle (10), to obtain a more industrially useful strain, outcrossing of independent strains with industrial characteristics has not been applicable. Therefore, development of alternative and diverse molecular breeding strategies is required. In this study, we demonstrate high-efficiency genome editing in *A. oryzae* via error-prone NHEJ by the transient expression of PtFg TALENs. We also demonstrate that PtFg TALEN-based genome editing via NHEJ repair is possible in the absence of the *ligD* gene involved in the final step of DNA NHEJ repair. This method may allow for targeted mutations to generate strains with specific phenotypes with industrial applications.

MATERIALS AND METHODS

Strains, media, and molecular biological techniques Standard *Escherichia coli* manipulations were performed as described previously (11). The *E. coli* strain DH5α (Nippon Gene Co., Ltd., Tokyo, Japan) was used for plasmid propagation. *A. oryzae* genomic DNA was isolated as described previously (12). *A. oryzae* RIB40 (National Research Institute of Brewing Stock Culture and ATCC42149) was used

* Corresponding author. Tel.: +81 82 420 0822; fax: +81 82 420 0807.
E-mail address: mizutani@nrib.go.jp (O. Mizutani).

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for the genome-sequencing project (13), and *A. oryzae ligD* disruptant (*ΔligD*::*ptrA*) derived from NS4 (14) and prepared previously (15) was used as the recipient strain for genome editing using PtFg-TALENs. These strains were grown in YPD complete medium (1% yeast extract, 2% polypeptone, 2% glucose), Czapek–Dox (CD) minimal medium (16), or CDME medium (17) for preparation of conidial suspensions. CDE medium (17) was used as the selection medium for *sC* gene transfer derivatives from *ΔligD*::*ptrA*. CDmSe medium (17) supplemented with 2% maltose instead of glucose to induce PtFg TALEN expression was used as the selection medium for *sC* mutant strains generated by PtFg TALENs in *A. oryzae*. CDSe medium (17) was used for the purification of *A. oryzae* transformants with *sC* mutation.

PtFg TALEN assembly method for *sC* gene mutation strain To construct the pPtFg TALEN final capture vectors adapted to *A. oryzae*, the *glaA*142 promoter and *agdA* terminator cassette were amplified by fusion PCR. All primers are described in Supplemental Table S1. For the first PCR, the *glaA*142 promoter with multiple cloning sites (*Apa*I, *Asc*I, and *Pac*I) was amplified from pNGA142 using glaA-1 and glaA-2 primers (Supplemental Table S1), and the *agdA* terminator with multiple cloning sites (*Sac*I, *Asc*I, and *Pac*I) was amplified from pNEN142 using agdA-1 and agdA-2 primers (Supplemental Table S1). The products of the first PCR were mixed and used as a template for the second PCR. The fusion PCR product was generated using glaA-1 and agdA-2 primers, and cloned between the *Apa*I and *Sac*I sites of the pGEM-T-easy vector, yielding pGEM-PglaA142-TagdA. The fragments containing N- and C-terminal domains, the *LacZ* cassette, and *Fok*I nuclease domain were transferred from pPtFgTALEN-HD, -NG, -NI and -NN (5) to the *Asc*I and *Pac*I sites of pGEM-PglaA142-TagdA, yielding *A. oryzae* expression vectors AopPtFgTALEN-HD, -NG, -NI, and -NN.

The plasmids pTALENsC-L and pTALENsC-R (Fig. 1B) carrying the PtFg TALEN cassette used for *sC* mutation were constructed according to the four-module assembly system described previously (4,5).

Construction of the *ΔligD*/*sC*+ in *A. oryzae* A fragment of the *sC* gene was obtained by PCR with primers AosCFw and AosCRv (Table S1, supplemental material) with *A. oryzae* RIB40 genomic DNA as the template. *A. oryzae ΔligD*::*ptrA* (15) was transformed with a fragment of the *sC* gene as previously described (18). *A. oryzae* transformants in which the *sC* fragment replaced mutated *sC* in *ΔligD*::*ptrA* were screened for sulfate prototrophy and purified by subculturing at least three times on CDE agar plates. These candidates were subjected to colony PCR as described previously (17) using the primer set AosCFw and AosCRv (Supplemental Table S1) with *A. oryzae* genomic DNA in cell lysate as the template. In addition, the PCR products were sequenced for confirmation of the change from Pro44 (CTT), the mutation site of the *sC* gene in *ΔligD*::*ptrA* strain, to Leu (CCT). The transformant with the correct sequence was named *ΔligD*/*sC*+. The genetic background of the *ΔligD*/*sC*+ strain is shown in Supplemental Table S2.

Transient transfection of PtFg TALENs The preparation of protoplasts and PEG transformation were based on previous methods (18,19) with slight modifications. Briefly, protoplasts (1×10^6 cells) of *A. oryzae* RIB40 and *ΔligD*/*sC*+ cells were mixed with 2 μg pTALENsC-L and 2 μg pTALENsC-R, then subjected to PEG transformation. The mixed sample was spread on CDmSe plates containing 0.8 M NaCl, and the plates were incubated at 30°C for 4–7 days.

A. oryzae transformants were purified by subculturing at least twice on CDSe agar plates. These candidates were subjected to colony PCR using primer sets sC conf Fw1 and Rv1, Fw2 and Rv2, or Fw3 and Rv3 (Table S1), with genomic DNA from cell lysate as the template. PCR products were sequenced to confirm the *sC* mutation caused by PtFg TALENs. Alternatively, genomic DNA was isolated from some candidates that did not yield amplification products by colony PCR, subjected to genomic PCR, and sequenced. The insertion sequence was analyzed using a BLAST search against the *A. oryzae* genome from the AspGD site (http://www.aspergillusgenome.org). If the sequence had no homology with the *A. oryzae* genome according to BLAST, it was analyzed by homology search against the pTALENsC plasmid using GENETYX software.

RESULTS

Genome editing via error-prone NHEJ repair using PtFg TALENs in *A. oryzae* RIB40 Arazoe et al. (5) reported that PtFg TALENs improved the efficiency of homologous recombination-mediated targeted gene replacement by up to 100% in the rice blast fungus *P. oryzae*. Therefore, we investigated the feasibility of PtFg TALEN-based genome editing via the error of NHEJ repair in *A. oryzae*. The *sC* gene encoding ATP sulfurylase conferring sulfate assimilation was used as the target because its mutation results in resistance to selenite (20). The TAL Effector Nucleotide Targeter 2.0 online tool (TALE-NT; https://tale-nt.cac.cornell.edu/) (21) was used to design TAL effectors. We selected a PtFg TALEN target region close to the active site of the ATP sulfurylase from regions provided by the TALE-NT (Fig. 1A). We next investigated whether the TAL effectors recognized off-target regions in the *A. oryzae* genome using the on-line tool Paired Target Finder (TALE-NT: https://tale-nt.cac.cornell.edu/node/add/talef-off-paired) (21,22) and found no potential off-targets in the *A. oryzae* genome. The PtFg TALEN genes were expressed under a high expression promoter, *A. oryzae glaA*142 (23), which is strongly induced by maltose. Using the PtFg TALEN system in *A. oryzae*, we constructed two plasmids harboring a PtFg TALEN expression cassette, pTALENsC-L and -R (Fig. 1B). The protoplasts of *A. oryzae* RIB40 were transiently transfected with pTALENsC-L and -R, and inoculated onto selenate plates containing 2% maltose (CDmSe plate) to screen for candidate strains with *sC* mutations caused by TALENs. Two transient transfections with pTALENsC-L and -R yielded 34 candidates. All candidates (TsC no. 1–34) grew when subcultured on CDSe plates. On the other hand, all 8 candidates obtained by transient transfection with only pTALENsC-L or no plasmids (as negative controls) failed to grow when subcultured on CDSe plates. To confirm whether a mutation in the *sC* gene occurred in the 34 subcultured candidates, colony-PCR was conducted using primers *sC* conf Fw1 and Rv1 (Fig. 2A). Amplification of an approximately 1.7-kb fragment was observed in 18 of the 34 subcultured candidates (data not shown). The obtained PCR products were sequenced, revealing deletions of several dozen bp (eight strains), deletions of several hundred bp

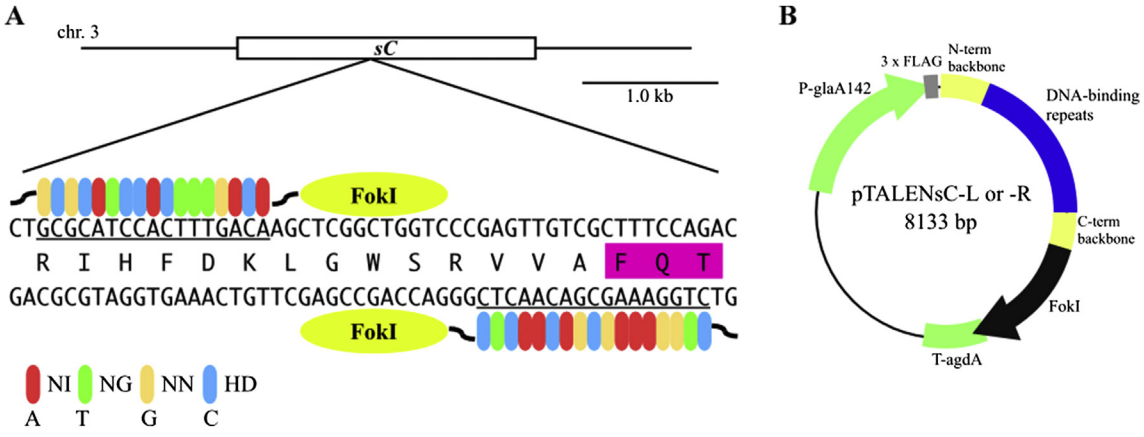

FIG. 1. Schematic diagram of the *sC*-targeting Platinum-Fungal (PtFg) TALENs and the expression plasmids for *A. oryzae*. (A) Schematic diagram of the *sC*-targeting region and construction of RVDs in the PtFg TALENs. *sC*-targeting sequences are underlined. TALE-repeat domains are colored to indicate the identity of RVD. (B) Construction of plasmids for PtFg TALEN expression. pTALENsC-L and -R harbor RVDs recognizing the left and right regions in the *sC*-targeting sequence, respectively.

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