Editorial

Highly efficient transgene-free targeted mutagenesis and single-stranded oligodeoxynucleotide-mediated precise knock-in in the industrial microalga *Euglena gracilis* using Cas9 ribonucleaseproteins

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Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system-based genome editing has been applied to a wide range of species, including economically important agricultural crops (Doudna and Charpentier, 2014). Genome editing by the direct delivery of ribonucleaseproteins (RNPs) offers various advantages compared to the conventional transgene-based method, including high efficiency, low time requirements, reduced off-target effects and low cytotoxicity (Jeon et al., 2017; Spicer and Molnar, 2018). Moreover, transgene-free genome-edited organisms may bypass current regulations for genetically modified organisms (Jeon et al., 2017; Spicer and Molnar, 2018), which is suitable for food and healthcare applications such as the molecular breeding of crops and microalgae. However, owing to the low mutation efficiency of screening-less and DNA-free RNP-based genome editing methods in microalgae (~1%) (Jeon et al., 2017; Spicer and Molnar, 2018), transgene-free genome editing technology needs to be further improved for practical applications.

*Euglena gracilis*, a unicellular photosynthesizing flagellate, is an industrially exploited microalga. *E. gracilis* is rich in nutrients and accumulates a crystallized β-1,3-glucan, paramylon (Inui et al., 2017), which has various bioactive functionalities (Suzuki, 2017). Therefore, mass-cultured *E. gracilis* is a commercial source for functional foods, feeds and cosmetics (Suzuki, 2017). Moreover, under anaerobic conditions, paramylon is degraded and converted to wax esters, which mainly consist of myristic acid (C14:0) and myristyl alcohol (C14:0) (Inui et al., 2017). As the wax ester of *E. gracilis* is easily reformed to a biofuel having a low freezing point, it is suitable as a source for biofuel production (Inui et al., 2017). Despite these promising features of *E. gracilis* as a renewable resource, the establishment of its effective targeted mutagenesis has been a long-standing challenge. In this study, we developed a highly efficient transgene-free targeted mutagenesis and single-stranded oligodeoxynucleotide (ssODN)-mediated knock-in in *E. gracilis* using Cas9 RNPs, which provides the first evidence that *E. gracilis* is a genome-editable organism.

To establish a transgene-free mutagenesis method based on Cas9 RNPs in *E. gracilis*, we designed two target sequences within the second exon of its glucan synthase-like 2 (*EgGSL2*, GenBank accession number: LC225615) gene (Figure 1a), which is putatively involved in paramylon biosynthesis (Tanaka et al., 2017). Each crRNA and tracrRNA was synthesized by Alt-R CRISPR-Cas9 system in Integrated DNA Technologies (IDT), and the same amounts of 100 μM solutions were used for annealing. Cas9 RNP complexes were prepared by mixing the cooled qRNA complex and Alt-R S.p. Cas9 Nuclease V3 (IDT, 62 μM solution) at a ratio of 3:2 (v/v) and incubating for 15 min at 20–25°C. Electroporation solution was prepared by mixing CM medium (pH 5.5) without sodium citrate and a filter-sterilized 0.3 mol/L sodium citrate and a filter-sterilized 0.3 mol/L sucrose solution at a ratio of 3:2 (v/v). *Euglena gracilis* Z strain provided by IAM (Tokyo, Japan) was cultured in KH medium (pH 5.5) on a rotary shaker (120 rpm) at 28°C under 50 μmol continuous light for 3 days, and 1 mL of the culture liquid was centrifuged at 400 × g for 30 s. *Euglena gracilis* pellets were washed once with electroporation solution and resuspended with electroporation solution at a concentration of 1 × 10⁶ cells/mL. For electroporation, 2 μL of RNP complex solution was added to 48 μL of *E. gracilis* suspension. We introduced Cas9 RNPs for the target regions in *EgGSL2* into *E. gracilis* cells by electroporation using a NEPA21 Super Electroporator with a 2-mm gap cuvette EC-002 (NEPPA GENE). Immediately after electroporation, 1 mL of KH medium was added, followed by transfer to a 12-well plate and culture on a rotary shaker (120 rpm) at 28°C under dark conditions (Figure 1b). After culture for 72 h, we confirmed that the *E. gracilis* cells exhibited fewer but larger paramylon granules with these two *EgGSL2*-targeting RNPs (Figure 1c). The average frequencies of
altered phenotypes for targets 1 and 2 were 71.3% and 80.6%, respectively (Figure 1d). We amplified the \textit{EgGSL2} locus using Gflex polymerase (Takara Bio) and detected mutagenesis by a T7 endonuclease I assay using the Alt-R Genome Editing Detection Kit (IDT) at 72 h after the direct delivery of Cas9 RNPs (Figure 1e). We also performed Sanger sequencing of the target regions amplified with KAPA HiFi HS ReadyMix (KAPA Biosystems) using an ion proton system (Thermo Fisher Scientific), we detected indel mutation rates of 77.7–86.8% and 88.8–90.1% at \textit{EgGSL2} targets 1 and 2, respectively, (Figure 1f) at 72 h after electroporation as assessed by the Cas-Analyzer software (Park et al., 2017). Additionally, the simultaneous introduction of two Cas9 RNPs targeting different sites, targets 1 and 2, into \textit{EgGSL2} yielded an approximately 200-bp deletion between the targets (Figure 1h,i), suggesting the ability to generate long deletion mutants of targeted regions in \textit{E. gracilis}.

To further advance Cas9 RNP-based genome editing in \textit{E. gracilis}, we demonstrated a precise ssODN-mediated knock-in experiment. We used the \textit{EgGSL2} target 2 Cas9 RNPs with ssODNs (1 μL of 200 μM stock solution, final concentration was 4 μM in electroporation solution) that include 50 nt upstream and downstream of the \textit{EgGSL2} cleavage site as homology arm. We used the \textit{EgGSL2} target 2 Cas9 RNPs with ssODNs (1 μL of 200 μM stock solution, final concentration was 4 μM in electroporation solution) that include 50 nt upstream and downstream of the \textit{EgGSL2} cleavage site as homology arm.
arms and a 42nt knock-in DNA fragment containing EcoRI, EcoRV and BamHI sites (Figure 1). We detected the effective knock-in events in ssODN-treated E. gracilis samples by digestion with these restriction enzymes (Figure 1k). We also performed Sanger sequencing of the knock-in target region using total E. gracilis cells at 72 h after electroporation and identified 24 out of 35 randomly cloned PCR products that had a precise knock-in sequence derived from our designed ssODN in the target site (Figure 1). These results demonstrate that ssODN-mediated knock-in experiments are sufficiently applicable to E. gracilis.

Our results demonstrate, for the first time, transgene-free targeted mutagenesis in E. gracilis, with an extremely high efficiency compared to that of previously reported genome editing attempts in other microalgae. We found maximum mutagenesis rates of more than 80% for EgGSL2 targeting (Figure 1q), which are significantly higher than the rates previously reported DNA-free targeted mutagenesis using Cas9 RNPs (~1%) or Cpf-1 RNPs with ssODN (~10%) in Chlamydomonas reinhardtii (Jeon et al., 2017; Spicer and Molnar, 2018), suggesting high editability of E. gracilis using RNP-based methods. We hypothesized that the lower genome editing efficiency in microalgae from the Viridiplantae subkingdom is related to their unique cell walls or cell surface structures, which potentially inhibit RNP penetration into cells (Jeon et al., 2017), compared to photosynthetic unicellular eukaryotes lacking cell walls, such as Euglena in the phylum Euglenozoa. In Trypanosoma cruzi, a parasitic protozoan belonging to Euglenozoa, Medeiros et al. recently demonstrated highly efficient genome editing with a small SaCas9 (124 kDa), instead of the commonly used SpCas9 (163 kDa) (Medeiros et al., 2017), suggesting that the efficiency of RNP-based genome editing is influenced by cell surface structures, as well as the physicochemical properties of RNP molecules. A high mutagenesis efficiency in the E. gracilis genome will accelerate its industrial use for foods and feeds. Using two gRNAs designed from adjacent sites on a chromosome, we also demonstrated the induction of a relatively large deletion (Figure 1l), which is useful for analysing the functions of non-protein-coding genetic codes such as long noncoding RNA genes and regulatory elements. We also demonstrated efficient knock-in using Cas9 RNPs with ssODNs as DNA donors (Figure 1j–l). Although it is necessary to investigate the effects of knock-in length and target site on the knock-in efficiency in a future work, this technique will lead to more precise and elaborate genome editing, as well as the creation of stable knock-in transformants for the elucidation of gene functions in E. gracilis.

The CRISPR-based technology will significantly accelerate gene discovery and molecular breeding in Euglena. Using our RNP-based genome editing approach in E. gracilis, we may be able to implement various CRISPR-derived technologies, such as CRISPR-mediated base editing and chromosome visualization (Doudna and Charpentier, 2014). CRISPR-based gene targeting also enables us to introduce functional mutations into duplicated genes in E. gracilis, which is thought to be a polyploid (Yamada et al., 2016), similar to polyploid crop plants such as wheat and cotton. Recent examples of improved lipid productivity in E. gracilis by ion-beam mutagenesis (Yamada et al., 2016) and RNA interference-based down-regulation of cellular signalling pathways (Kimura and Ishikawa, 2018) suggest that the targeting of negative regulator genes is a promising approach to identify genetic factors involved in lipid productivity in E. gracilis and to stably improve this trait. Our RNP-based genome editing method in E. gracilis also provides a breakthrough to elucidate the functions of genes in other euglenids, the secondary plastid-containing group in the Excavata supergroup, and opens up new avenues for improving their industrially important traits to promote sustainability by bio-based material production.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

TN conceived the study and designed the experiments. TN and YU performed experiments and analysed the data. KI performed the bioinformatics analysis. KY, OI and KS provided the biological materials. KM supervised the project, and TN and KM wrote the manuscript. All authors read and approved the final manuscript.

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