CRISPR/Cas-Mediated Targeted Mutagenesis in *Daphnia magna*

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**Abstract**

The water flea *Daphnia magna* has been used as an animal model in ecology, evolution, and environmental sciences. Thanks to the recent progress in *Daphnia* genomics, genetic information such as the draft genome sequence and expressed sequence tags (ESTs) is now available. To investigate the relationship between phenotypes and the available genetic information about *Daphnia*, some gene manipulation methods have been developed. However, a technique to induce targeted mutagenesis into *Daphnia* genome remains elusive. To overcome this problem, we focused on an emerging genome editing technique mediated by the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system to introduce genomic mutations. In this study, we targeted a functionally conserved regulator of eye development, the *eyeless* gene in *D. magna*. When we injected Cas9 mRNAs and *eyeless*-targeting guide RNAs into eggs, 18–47% of the survived juveniles exhibited abnormal eye morphology. After maturation, up to 8.2% of the adults produced progeny with deformed eyes, which carried mutations in the *eyeless* loci. These results showed that CRISPR/Cas system could introduce heritable mutations into the endogenous *eyeless* gene in *D. magna*. This is the first report of a targeted gene knockout technique in *Daphnia* and will be useful in uncovering *Daphnia* gene functions.

**Introduction**

The water flea *Daphnia magna* is a planktonic crustacean ubiquitously found in the fresh water environment. It has been used as a model organism in ecology and toxicology because it is sensitive to artificial chemicals and environmental changes [1]. Moreover, researchers find it interesting that *Daphnia* can switch their reproduction mode from asexual to sexual in response to environmental stimuli [2]. Recent progress in genomics involved analyses of expressed sequence tags (ESTs) [3] and the draft genome sequence of *D. magna*. In addition, the genome sequence of a related organism, *D. pulex*, has recently been completed [4]. Therefore, a vast amount of genetic information on *Daphnia* is now available. To investigate the relationship between available genetic information and phenotypes, gene manipulation tools such as RNA interference (RNAi) and non-homologous integration with plasmid DNA have been developed in *D. magna* [5,6]. However, there is still no technique to induce inheritable targeted gene disruptions.

The clustered regularly interspaced palindromic repeats/CRISPR-associated (CRISPR/Cas) system is a recently developed tool to induce targeted mutagenesis. CRISPR/Cas was initially identified as the bacterial immune system to bacteriophages [7]. In this system, a CRISPR RNA (crRNA) interacts with a transactivating CRISPR RNA (tracrRNA) and forms the tracrRNA-crRNA duplex, which acts as a guide RNA (gRNA) that directs the endonuclease Cas9 to its cognate target DNA and induces double-strand breaks (DSBs) [8]. Importantly, the cleavage site is often imperfectly repaired by the error-prone non-homologous end-joining (NHEJ) mechanism, resulting in gene disruptions through the introduction of small insertions or deletions (in-dels). Recent studies reported that a single chimeric RNA, a crRNA fused with a tracrRNA, could also function as a gRNA [8]. The approximately 20 bp targetable sites are limited by the requirement for the protospacer adjacent motif (PAM; 5’-NGG-3’) at their 3’ end [8]. Further constraint that target sites start with a GG dinucleotide is often required when gRNAs are synthesized in *vitro* by T7 polymerase. However, recent studies suggested some alternatives to circumvent the latter limitation without significant reduction of cleavage efficiency [9–11]. Therefore, to induce targeted mutagenesis, co-expression of the customized gRNA with the Cas9 nuclease has been used in various organisms [9–25] (Figure 1A). Compared with the other targeted mutagenesis tools such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), the experimental design of CRISPR/Cas is remarkably simple and rapid [26]. However, nobody has applied these emerging techniques to *Daphnia*.

The mammalian *pax6* gene ortholog is a functionally conserved regulator for eye development that has been conserved from invertebrates to vertebrates. It encodes a transcription factor with two DNA-binding domains: paired box and homeobox domains. In *Drosophila*, a mammalian *pax6* homolog was originally mapped in the *eyeless* (*ey*) locus [27], whose mutants show abnormal eye
morphogenesis, resulting in complete or partial loss of eye as well as mutations in the mouse pax6 gene [27–31]. Animals with abnormal eyes could be easily distinguished from normal ones in appearance, suggesting that pax6 ortholog would be a useful model target gene for targeted mutagenesis as previously used for TALEN-mediated mutagenesis [32].

In the present study, D. magna ey gene was partially cloned and its role in eye development was confirmed by RNAi, thus prompting us to use ey as a target for CRISPR/Cas-mediated mutagenesis. We showed that the CRISPR/Cas system efficiently introduces heritable in-del mutations into the ey loci of the D. magna genome, resulting in deformations of the compound eye. In addition, undesired mutations (off-target mutations) were not detected in the genomes of the ey-deficient daphniids. Thus, we concluded that the CRISPR/Cas system is a powerful tool for modifying targeted genomic sites and can facilitate studies on the functional genomics of D. magna.

Results

Functional analyses of D. magna eyeless (Dma-ey) gene

To utilize the ey gene as a marker gene for targeted mutagenesis, we searched for mammalian pax6 orthologs in the D. magna genome and found the ey gene in addition to its paralog, twin of eyeless (toy) gene, both of which are conserved among several arthropods [33]. We named D. magna ey as Dma-ey in this study. By using the D. magna genome database, the Dma-ey gene was predicted to consist of 13 exons (Figure 1B). Reverse transcription-PCR (RT-PCR) of the Dma-ey gene using a primer set encompassing the homeobox as well as sequencing of the PCR fragments revealed that Dma-ey is expressed in D. magna (Figure S1).

Next, we tested whether the Dma-ey gene is required for compound eye development by RNAi. We designed siRNA within the homeobox of Dma-ey. A compound eye in a wild-type daphniid is located at the anterior parietal edge and roughly spherical in shape (Figure 2A, left), whereas daphniids injected with 100 μM...
siRNA had deformed compound eyes, which were located at the inner side and could not build a precise spherical shape (Figure 2A, right). Afterward, we call this phenotype as deformed eye in this study. Taken together, our results indicated that the eyeless gene is functionally conserved in D. magna, suggesting that it would be a useful marker gene for subsequent knockout experiments using the CRISPR/Cas system.

Dma-ey gene disruption by CRISPR/Cas system

To test whether the CRISPR/Cas system could induce targeted mutagenesis in D. magna, we attempted to introduce in-del mutations into the homeobox domain of the Dma-ey locus. Previous reports described ey-deficient Drosophila flies whose mutated ey allele lacked all C-terminal domains including the homeobox domain [31]. Thus, we hypothesized that Dma-ey-deficient daphniids can be generated by inducing frameshifts at an earlier part of the homeobox domain. To target the homeobox region, we used two gRNAs, gRNA-1 and gRNA-2, which were designed to bind the sense strand from exon 8 and the anti-sense strand from exon 10, respectively (Figures 1B and 2B). Because a previous report suggested that co-injection of multiple gRNAs increased mutation efficiency [17], we coinjected two gRNAs together with the Cas9 mRNA that contained the untranslated regions (UTRs) of D. magna vasa (Dmava) gene, which is exclusively expressed in D. magna germ cells [34]. We tested three and two different concentrations of Cas9 mRNA (500; 1,000; 2,000 ng/mL) and gRNAs (50; 150 ng/mL each), respectively (Table 1). At the first instar juvenile stage, 61–78% of the injected embryos survived. In addition, as expected, 18–47% of the surviving juveniles developed deformed eye phenotypes without significant
we collected deformed eye G1 progenies from 5 induce heritable mutations in the endogenous mutations were found in normal eye G1 progenies (data not gRNA-targeted sites (Figure 2B). In contrast, monoallelic in-del deformed eye G1 progenies had biallelic in-del mutations around respectively (Table 1). Genomic PCR products encompassing the different founder lines and extracted their genomic DNAs somatic cells but also in germ line cells.

Table 1. Mutation frequencies induced by microinjection using various concentrations of Cas9 mRNA/gRNA mix.

| RNA concentration (ng/μL) | Embryos | Juveniles | Adults |
|---------------------------|---------|-----------|--------|
| gRNA mix Cas9 mRNA | Injected | Surviving | Deformed eye | Surviving | Founder lines |
| 50 each 500 | 77 | 59/77 (77%) | 28/59 (47%) | 49/77 (64%) | *4/49 (8.2%) |
| 1,000 | 121 | 90/121 (74%) | 16/90 (18%) | 81/121 (67%) | 5/81 (6.2%) |
| 2,000 | 113 | 75/113 (66%) | 29/75 (39%) | 61/113 (54%) | 0/61 (0%) |
| 150 each 500 | 98 | 70/98 (71%) | 19/70 (27%) | 60/98 (61%) | 2/60 (3.3%) |
| 1,000 | 86 | 67/86 (78%) | 20/67 (30%) | 60/86 (70%) | *2/60 (3.3%) |
| 2,000 | 64 | 39/64 (61%) | 8/39 (21%) | 38/64 (59%) | *1/38 (2.6%) |

*Mutant lines subjected to sequencing of Dma-ey loci. In Figure 2B, two mutants named m1 and m2 were from 4 mutants injected with 50 ng/μL each of gRNA and 500 ng/μL Cas9 mRNA (50 each, 500), m3 and m4 from 2 mutants (150 each, 1,000), and m5 from 1 mutant (150 each, 2,000).

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Discussion

Here, we described a simple, rapid, and efficient technique for inducing target mutagenesis into an endogenous locus of the D. magna genome by using a CRISPR/Cas system, which will enable us to perform high-throughput functional analyses of D. magna genes. This system would contribute to overcoming two limitations in previous Daphnia reverse genetics studies using RNAi by microinjection of dsRNAs into eggs [5]: (1) incapability to induce null phenotypes and (2) transient nature of gene manipulation. The knockout Daphnia lines allow us to observe the loss-of-function phenotypes throughout life cycle, which will undoubtedly advance our understanding of D. magna gene functions.

We coinjected pairs of gRNAs, gRNA-1, and gRNA-2, which targeted exons 8 and 10 of Dma-ey, together with Cas9 mRNA. We found that all five Dma-ey G1 mutants with deformed eyes have biallelic mutations. This simple and efficient induction of biallelic mutations seems to be beneficial for researchers studying Daphnia that usually produce parthenogenetic females. For crossing to establish homozygous mutants in Daphnia, we have to induce the production of males and sexual females that lay haploid eggs by stimulating the parthenogenetic females with environmental cues such as shortened photoperiod, lack of food, and/or increased population density, which makes the crossing procedure laborious and time consuming. Ability of the CRISPR/Cas system to induce biallelic mutations would significantly improve the genetics of parthenogenetic organisms, including Daphnia.

In the current study, no founder animals were observed when we used 2,000 ng/μL of Cas9 mRNA and 50 ng/μL each of gRNA for injection. One possible explanation for this result is the aggregation of free abundant Cas9 proteins which prevents them from forming a complex with gRNAs. This interpretation seemingly corresponds with our data since we could establish one founder animal by injection of the same concentration of Cas9 mRNA with a higher concentration of gRNAs (see Table 1). Although the reason is not clear based on our current data, the proportion of Cas9/gRNA used should be important for the activity of CRISPR/Cas systems.

We further analyzed if Dma-ey-deficient mutants had undesired mutations (off-target mutations). Pioneering works suggested that genomic sites have mismatches fewer than 5 bp with PAM (NGG at 3’ end) sequence could be cleaved (= potential off-target sites) [19,35]. By using a BLASTn search on the D. magna genome database, we looked for potential off-target sites and found that gRNA-1 had 4 potential off-target sites, whereas gRNA-2 did not (Table 2). To test if Dma-ey-deficient mutants have off-target in-del mutations, we designed a primer set to amplify each potential off-target site of gRNA-1 and performed PCRs using genomic DNAs from 5 different G1 mutants subjected to previous analyses of in-del mutations in the Dma-ey locus. Consequently, no off-target mutation was observed. These results suggested that the CRISPR/Cas-mediated targeted mutagenesis approach was highly specific.

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Of the 14 deformed eye mutants established in this study, one was viable. Biallelic Dma-ey mutations should give rise to lethality because previous reports described that homozygous mutation in mammalian Pax6 and its homolog ey were lethal to mice and flies, respectively [29,31]. Our sequence data showed that the viable mutant lost the 877 bp region including the entire homeobox.
domain on the Dma-ey allele but it retained the correct reading frame of the remaining C-terminal region (Figure 2B, m5), whereas coding frames of the other sequenced alleles were shifted, hence all the sequences from the homeobox domain to the C-terminal region were lost (Figure 2B, m1 to m4). This might account for the difference in the mutants’ viability. Previous works described the PAX6 protein as having a conserved C-terminal domain which, follows homeobox domain, is rich in proline (P), serine (S), and threonine (T) residues, and which mediates activation of PAX6 protein via phosphorylation [36,37,30]. Therefore, the presence of the C-terminal activation domain on the 877 bp deleted Dma-ey allele might contribute to the viability of the deformed eye mutant D. magna.

Of the 10 alleles from five deformed eye mutants analyzed in this study, nine had indel mutations in one of the two target sites (Figure 2B). The patterns of these mutations were consistent with those induced by Cas9-based cleavage in the other animals. Interestingly, one allele had an 877-bp deletion spanning both target sites, suggesting the possibility that concurrent DSBs at two distantly target sites for gRNAs induced this large deletion, as reported in previous studies [9,12,17,22].

CRISPR-based genome engineering will also provide novel approaches to integrate foreign DNAs into the D. magna genome. DSB sites are predominately repaired by either NHEJ or homologous recombination (HR) [39]. Double-strand cleavage has been shown to facilitate the rate of homologous gene targeting at the cleaved site [40]. Successes of targeted knock-ins have been reported through the co-introduction of CRISPR components and exogenous DNAs such as plasmids or single-strand oligoDNAs (ssODNs) that have a homologous region to the cleavage site [40,36,37,19,25]. This approach will enable us to induce targeted knock-in of DNA fragments such as integrase-targeting sequences or epitope tag-coding sequences, even in D. magna.

Thus, CRISPR/Cas system-mediated genome editing technique described here will definitely accelerate the development of Daphnia functional genomics.

### Materials and Methods

**Daphnia strain and culture conditions**

The D. magna strain (NIES clone) was obtained from the National Institute for Environmental Studies (NIES, Tsukuba, Japan) and cultured under laboratory conditions for many generations. To minimize variations in maternal effects that may follow homeobox domain, is rich in proline (P), serine (S), and threonine (T) residues, and which mediates activation of PAX6 protein via phosphorylation [36,37,30]. Therefore, the presence of the C-terminal activation domain on the 877 bp deleted Dma-ey allele might contribute to the viability of the deformed eye mutant D. magna.

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### Construction of RNA expression vectors

To generate the Cas9 expression vector pCS-Dmavas-Cas9, the Cas9 ORF was amplified using the plasmid MLM3613 (Addgene plasmid 42251, [18]) as a PCR template. DNA fragments of the 5’ and 3’ UTRs from the Dmavas gene (Accession: AB193324.1) were obtained by PCR using cDNAs of the NIES strain. These PCR products were simultaneously cloned into the downstream region of the SP6 promoter of pCS+ vector using the In-Fusion PCR cloning kit (Clontech, California, USA) [42]. To generate the gRNA expression vector pDR274-Dma-ey, the plasmid DR274 (Addgene plasmid 42250, [18]) was digested with Bsal (NEW ENGLAND BioLabs, Connecticut, USA) followed by dephosphorylation with Antarctic Phosphatase (NEW ENGLAND BioLabs, Connecticut, USA). A pair of Dma-ey targeting oligonucleotides was annealed and then ligated into the linearized pDR274 vector using a ligation mix (TaKaRa Bio, Shiga, Japan). The genomic target sites and sequences of the oligonucleotides constructed in this study are listed in Tables S1 and S2. All PCRs in this section were performed with PrimeSTAR (Takara Bio, Shiga, Japan).

### In vitro RNA synthesis

siRNAs for knocking down the Dma-ey gene were designed by using Block-iT RNAi Designer (Life Technologies, California, USA) and two nucleotides dTdT were added to each 3’ end of the siRNA strand. The sequences of the siRNA are listed in Table S1. For the syntheses of Cas9 mRNAs, templates with T7 promoter were amplified by PCR from the pCS-Dmavas-Cas9. The primer sequences for the PGR are shown in Table S2. Amplified PCR fragments were subjected to in vitro transcription with the mMessage mMachine T7 kit (Life Technologies, California, USA). Poly (A) tails were attached to capped Cas9 RNAs by using a Poly(A) Tailing Kit (Life Technologies, California, USA), following the manufacturer’s instructions. The synthesized mRNAs were column purified using mini Quick Spin RNA columns (Roche diagnostics GmbH, Mannheim, Germany) followed by phenol/chloroform extraction, ethanol precipitation, and dissolution in DNase/RNase-free water (Life Technologies, California, USA).
For the synthesis of gRNAs, pDR274-Dma-ey vectors were digested by DdI and purified by phenol/chloroform extraction. DdI-digested DNA fragments were used as templates for in vitro transcription with the mMessage mMachine T7 kit, followed by column purification with mini Quick Spin RNA columns, phenol/chloroform extraction, ethanol precipitation, and dissolution in DNase/RNase-free water.

Microinjection

*In vitro* synthesized RNAs were injected into *Daphnia* eggs according to established procedures [5]. Briefly, eggs were collected from daphnids within 2–3 weeks of age just after ovulation and placed in ice-chilled M4 medium containing 80 mM sucrose (M4-sucrose). The synthesized RNAs were injected through a glass needle with N₂ gas pressure. The injection volume was approximately 0.2 nL. Finally, an injected egg was transferred into each well of a 96-well plate filled with 100 µL of M4-sucrose. Microinjections were carried out within an hour after ovulation.

PCR amplification of target loci

To characterize Cas9-induced mutations at the molecular level, target loci were amplified by PCR using genomic DNA extracted from deformed eye daphnids. Genomic DNA was extracted from single daphnids by homogenization in 90 µL of 50 mM NaOH with zirconia beads. The lysate was heated at 95°C for 10 min and then neutralized with 10 µL of 1 M Tris-HCl (pH 7.5). This crude DNA extract was centrifuged at 12,000 rpm for 5 min prior to being used as a template for genomic PCR. The targeted genomic region was amplified by PCR with KOD plus (TOYOBO, Osaka, Japan). The PCR products were analyzed by agarose gel electrophoresis and DNA sequencing. The primers used for PCR and DNA sequencing are listed in Tables S3 and S4.

**Supporting Information**

**Figure S1** Results of RT-PCR for Dma-ey and cloned partial cDNA sequence. (A) Electrophoresis of PCR products. M: 100-bp ladder marker (TOYOBO, Osaka, Japan); G: genomic PCR product as positive control of PCR, RT+: RT-PCR product amplified from reverse-transcribed cDNAs, RT-: RT-PCR product amplified from total RNAs without reverse transcription. (B) Sequence of partially cloned Dma-ey cDNA.

**Table S1** Oligonucleotides used for verification of off-target mutations by sequencing.

**Table S2** Oligonucleotides used as *in vitro* transcription templates.

**Table S3** Oligonucleotides used for the verification of cutting sites by sequencing.

**Table S4** Oligonucleotides used for verification of off-target mutations by sequencing.

**Author Contributions**

Conceived and designed the experiments: TN YK TM HW. Performed the experiments: TN. Analyzed the data: TN HW. Contributed reagents/materials/analysis tools: TN YK HW. Wrote the paper: TN YK HW.

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