Development of transgenic *Daphnia magna* for visualizing homology-directed repair of DNA

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In the crustacean *Daphnia magna*, studying homology-directed repair (HDR) is important to understand genome maintenance during parthenogenesis, effects of environmental toxicants on the genome, and improvement of HDR-mediated genome editing. Here we developed a transgenic *D. magna* that expresses green fluorescence protein (GFP) upon HDR occurrence. We utilized the previously established reporter plasmid named DR-GFP that has a mutated *eGFP* gene (*SceGFP*) and the tandemly located donor *GFP* gene fragment (*iGFP*). Upon double-strand break (DSB) introduction on *SceGFP*, the *iGFP* gene fragment acts as the HDR template and restores functional *eGFP* expression. We customized this reporter plasmid to allow bicistronic expression of the *mCherry* gene under the control of the *D. magna* EF1α-1 promoter/enhancer. By CRISPR/Cas-mediated knock-in of this plasmid via non-homologous joining, we generated the transgenic *D. magna* that expresses *mCherry* ubiquitously, suggesting that the DR-GFP reporter gene is expressed in most cells. Introducing DSB on the *SceGFP* resulted in *eGFP* expression and this HDR event could be detected by fluorescence, genomic PCR, and quantitative reverse-transcription PCR, suggesting this line could be used for evaluating HDR. The established reporter line might expand our understanding of the HDR mechanism and also improve the HDR-based gene-editing system in this species.

Genomes are threatened by endogenously generated chemicals like reactive oxygen species and exogenous compounds such as mutagenic agents and radiation1, which can lead to DNA double-strand breaks (DSBs). To ensure genetic stability and cellular viability, repairing the DSBs is essential. The DNA repair mainly occurs through non-homologous end joining (NHEJ) and homology-directed repair (HDR)2. The NHEJ leads to ligation of the two ends of the DNA strand during which insertion or deletion of nucleotides (indels) can often occur at the cleavage site. The HDR repairs the DSBs by using information copied from undamaged DNA that has an identical or homologous sequence (homology)3. This homology-directed repair system can be divided into four sub-pathways based on the mechanistic difference: double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA), break-induced replication (BIR), and single-strand annealing (SSA)4. First, in the DSBR, the formation of an intermediate structure with the Holliday junctions (HJs) leads to the generation of crossover and non-crossover products4. Second, the SDSA exclusively generates the non-crossover products due to the lack of formation of the HJ structure5. Third, when only one ended DSB site has a sequence similar to that of the template, BIR occurs for non-reciprocal translocation of genetic information from the template strand6. Fourth, when the DSB is induced between the tandem repeats of the highly homologous regions, the SSA repairs the DNA by pairing the homologous region followed by deletion of unpaired DNA and the intervening region7. In the mitotically proliferating cells, the SSA is known to be the most common among the HDR sub-pathways8.

The HDR also plays an important role in the field of genome editing due to its nature of high fidelity and accuracy. Unlike NHEJ, HDR avoids multiple integrations of a donor DNA and indels between transgene and surrounding genomic region. Thus, following DSB using the programmable nuclease for instance TALEN or CRISPR-Cas, a precise genome modification such as the codon replacements or the seamless integration of the fluorescent reporter gene can be achieved by using a donor DNA flanked with right and left locus-specific homology arms as a template9,10.

The water flea *Daphnia magna* is a small freshwater crustacean found in broad continents such as Europe, the Middle East, Central Asia, Africa, and North America11. The genus *Daphnia* reproduces by parthenogenesis under favorable environmental conditions but switches it to sexual reproduction in response to environmental
stimuli such as shortened photoperiod, a lack of food, and/or increased population density\(^2\). The sequenced genome of *Daphnia* reveals highly duplicated genes, resulting in tandem gene clusters\(^13\). These tandem clusters may serve as a template for HDR-based repair to attenuate the effect of deleterious mutations during the parthenogenetic cycle, which suggests that *Daphnia* may have a unique HDR mechanism.

*Daphnia magna* occupies an important position in the freshwater food chain and is highly sensitive to chemicals, which makes this species a model in environmental and toxicological studies. The effects of genotoxicants have been investigated at the phenotypic level\(^14,15\). To understand their actions at the molecular level, it is important to study the DNA repair mechanism in this species. In the field of genome editing, the HDR-based knock-in of the exogenous DNA fragments has been reported in *D. magna*\(^16\) as well as NHEJ-mediated knock-in\(^17,18\). The HDR-based knock-in efficiency was low probably due to competition with the NHEJ pathway. To test this hypothesis, disruption of DNA ligase IV, which is the conserved component of the NHEJ pathway, has been attempted\(^16\) However, its effect was not fully evaluated due to the lack of a method for quantifying the HDR event in vivo. Therefore, a system to evaluate and quantify the HDR event is a necessity.

Fluorescence live imaging of the HDR event is essential not only for investigating how and where the genome integrity is maintained in living organisms but also for evaluating the HDR activity by manipulating the components for DNA repair. The direct repeat GFP (DR-GFP) reporter assay has been established for fluorescence-based visualization of the HDR activity\(^19\). The DR-GFP reporter system is composed of two mutated eGFP genes (Fig. 1, Genotype). The upstream eGFP gene named SceGFP contains a recognition site of the rare-cutting I-SceI restriction enzyme. This recognition site contains two in-frame stop codons to terminate the protein expression. At downstream of the SceGFP, there is another mutated eGFP fragment termed internal GFP or iGFP that is an 812-bp internal GFP fragment. The HDR event can be detected by introducing a double-strand break (DSB) with I-SceI in the inactive SceGFP gene. The cleavage site will be repaired by HDR using iGFP as the template. Among the HDR sub-pathways, this DR-GFP system can visualize the non-crossover events that are mediated by the DSBR and SDSA\(^20\), suggesting that this reporter can visualize the major HDR events spatiotemporally in vivo. This reporter has been applied to study the factors that contribute to HDR in mouse\(^21\) and to study the role of a transcriptional repressor protein in HDR using *C. elegans* models\(^22\).

Here we integrated the DR-GFP reporter system in the *D. magna* genome (Fig. 1, Genotype). We confirmed its functionality by introducing DSBs at the SceGFP region with the CRISPR/Cas9 system and detecting the eGFP signal spatiotemporally (Fig. 1, Phenotype). Furthermore, we could detect the repaired eGFP gene by genomic PCR and qPCR, which adds merit to this system to be utilized for the evaluation of the HDR event. By applying different stimuli (Fig. 1, Stimuli), the established transgenic *Daphnia* might contribute to various scientific fields such as ecotoxicology, genome editing, and evolutionary biology.

**Materials and methods**

*Daphnia magna* strain and culture condition. Wild type *D. magna* (NIES clone) was obtained from the National Institute of Environmental Studies (NIES, Tsukuba, Japan) and has been maintained in the laboratory for many generations. The *D. magna* was cultured under the following conditions: 80 juveniles (less than 24 h old) were collected and cultured in 5 L Artificial *Daphnia* Medium (ADaM)\(^23\) at temperature 22–24 °C, under 16 h/8 h of light/dark photoperiod. *D. magna* were fed daily with 8 × 10° cells of *Chlorella vulgaris* (Oita Medaka Biyori, Oita, Japan) and 3 mg of baker’s yeast (Marusan Pantry, Ehime, Japan) during the first week. Later, juvenile
niles were removed daily and amounts of chlorella and yeast extract were doubled. The culture medium was changed once a week.

**Customization of reporter plasmid.** To visualize the HDR by fluorescence in *D. magna*, a reporter donor plasmid pEF1α-1::mCherry-2A-DR-GFP was constructed by customizing the previously established pDRGFP plasmid (Addgene No.26475)\(^1^9\) (Fig. 2A, B). To allow ubiquitous expression in *D. magna*, the original chicken β-actin promoter was replaced with a 2.3 kb of *D. magna* elongation factor 1α-1 (EF1α-1) promoter/enhancer, including the transcription start site, the complete first intron, and part of the second exon with a start codon\(^1^7,2^4\). In addition, to recapitulate EF1α-1 endogenous expression, a full-length EF1α-1 3′ UTR was added downstream of the reporter. We retained the original two mutated eGFP fragments (SceGFP and iGFP) along with their nuclear localization sequence to distinguish the eGFP-expressing cells individually.

**DR-GFP reporter will function when the DSB is introduced in the I-SceI site.** By SDSA or non-crossover DSBR subpathway of HDR, SceGFP will use iGFP as a repair template resulting in the functional eGFP expression (Fig. 2C). A red fluorescent protein gene *mCherry* ORF was fused upstream of the DR-GFP via a sequence encoding *Thosea asigna* virus 2A (T2A) peptide indicated in the yellow box. SceGFP contains a recognition site of the 18 bp I-SceI restriction enzyme and in-frame two stop codons indicated in the blue underline and red letter respectively. SceI gRNA was designed to correspond with the I-SceI recognition site (italic) upstream of the PAM sequence (small letter). The cleavage site of SceI gRNA was indicated by a black triangle. (B) The alignment between eGFP, iGFP, and SceGFP sequences. Blue areas indicate the I-SceI site. (C) The diagram of the DR-GFP system for reporting HDR events. Double-strand break (DSB) is introduced in the I-SceI site by the Cas9-gRNA complex. Following homology-directed repair (HDR) occurrence, iGFP will serve as a repair template, leading to SceGFP repair indicated by eGFP expression green box.
target sequence\textsuperscript{26,27} was added. The complete nucleotide sequence of the customized DR-GFP reporter and the deduced amino sequence is provided in Supplementary Figure S1. All assemblies were performed using GeneArt Cloning & Assembly (Invitrogen, Carlsbad, USA). The constructed donor plasmid was purified using FastGene Plasmid Mini Kit (Nippon Genetics, Tokyo, Japan) and sequenced. The donor plasmid used for microinjection was purified using PureYield Miniprep (Promega, Madison, USA) followed by phenol–chloroform purification, two times ethanol washing, and was re-suspended with ultrapure water (Invitrogen).

**In vitro RNA synthesis.** Guide RNAs (gRNAs) were synthesized using a cloning-free method from PCR-amplified template DNA as previously described\textsuperscript{27}. The sense synthetic oligonucleotide containing a T7 promoter sequence, and the first 20 nt of the Cas9 binding scaffold were shown in Table 1. gRNAs were synthesized using the MegaScript T7 Transcription Kit (Invitrogen), purified using Roche Mini Quick Spin RNA Column (Roche, Mannheim, Germany) followed by phenol/chloroform extraction, ethanol precipitation.

For Cas9 mRNA synthesis, a template DNA containing T7 promoter sequence was PCR amplified from pCS-Dmavas-Cas9\textsuperscript{28}. Capped mRNA synthesis and poly(A) tail addition were performed using mMessage mMachine T7 kit and Poly(A) Tailing Kit (Invitrogen) respectively. Synthesized mRNA was column purified, followed by phenol/chloroform extraction and ethanol precipitation. mRNA integrity and the addition of poly(A) tails were confirmed by denaturing formaldehyde gel electrophoresis.

**Generation of HDR reporter transgenic *Daphnia magna.*** For the generation of the DR-GFP line, the customized DR-GFP reporter plasmid was integrated into *D. magna* genome by utilizing the CRISPR/Cas-mediated knock-in via non-homologous end-joining\textsuperscript{17}. Microinjection into *Daphnia* embryos was performed following an established protocol using the *S. pyrogenes*-originated Cas9 proteins\textsuperscript{17,29}. The Cas9 proteins were expressed in *E. coli* strain BL21 (DE3) and purified following established protocol\textsuperscript{10}. Fifty nanograms per microliter of purified donor plasmid was co-injected with 2 μM scarlet targeting gRNA, and 1 μM Cas9 protein. Shortly before the microinjection, Cas9 protein and gRNA were incubated at 37 °C for 5 min to form a ribonucleaseprotein (RNP) complex. Microinjection was performed within two hours after the preparation of the solution. After injection, the intact eggs were transferred, cultured individually in a sterile 96 well plate, and put in an incubator at 22 °C with 16 h/8 h of light/dark photoperiod for 3 days. Each well of the 96 well-plate was filled with 100 μL of M4-sucrose. Transgenic candidates were screened based on the mCherry expression in the ovary of injected embryos (G0). mCherry expressing offspring were cultured and genotyping was performed using the second generation offsprings (G2).

**Genotyping.** *Daphniids* were collected and homogenized in 500 μL lysis buffer (50 mM Tris–HCl pH 7.5, 20 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS) using MicroSmash homogenizer (TOMY, Tokyo, Japan) at 3000 rpm for 1.5 min with the presence of 0.15 mg/mL Proteinase K (Nacalai Tesque, Kyoto, Japan). The homogenized *daphniids* (lysate) were shaken overnight at 55 °C, 450 rpm using an incubator shaker (Bioshaker M-BR-022UP, TAITEC, Tokyo, Japan). To obtain genomic DNA (gDNA), the lysate was purified using phenol/chloroform extraction, precipitated with isopropanol, rinsed twice with 70% ethanol, and dissolved in 50 μL TE buffer before being used as a template for genomic PCR. The PCR was performed by using an Ex Taq Hot-Start DNA polymerase (TäKaRa) with primer sets amplifying the target region as described in Table 2.

| No | gRNA target         | Sense oligonucleotide                  |
|----|---------------------|----------------------------------------|
| 1  | Scarlet (st)        | 5′-GAATAATACGACTCACTATAAACGGCATTTGTAAGCCTAGAAA-3′ |
| 2  | Distal-less (Dll)   | 5′-GAATAATACGACTCACTATAAACGGCATTTGTAAGCCTAGAAA-3′ |
| 3  | SceI                | 5′-GAATAATACGACTCACTATAAACGGCATTTGTAAGCCTAGAAA-3′ |

Table 1. The sense sequence of the oligonucleotide for guide RNA synthesis. A T7 promoter, a targeting sequence, and the first 20 bp of the Cas9 binding scaffold sequence were indicated with bold letters, underline, and italic letters respectively.

To confirm whether Cas9 was active during microinjection, we also co-injected the SceI gRNA with another gRNA targeting *Distal-less (Dll)* gene. Previously RNAi-mediated knockdown of *Dll* in embryos of *D. magna* led to a distinct phenotype “truncation of second antennae” and the level of this truncation corresponded to the degree of impairment of this gene\textsuperscript{26}. The *Dll* gRNA was designed to target the upstream of the homeodomain region in exon 2 (Supplementary Figure S2), as this region is highly conserved among arthropods\textsuperscript{29} and considered important for *Dll* function\textsuperscript{1,2}.

The phenotypes of the second antennae of the injected embryos were observed 48 h post-injection (hpi) and categorized as normal, mild, medium, or strong truncation following the previous study. In normal phenotype, second antennae consist of a protopodite (*P*), carrying a dorsal and ventral ramus. Each ramus has three segments, Terminal (*T*), Middle (*M*), and Basal (*B*). There is an additional small wedge-shaped segment (*w*) between
after the HDR. The mCherry filter (Leica Microsystems) was used to screen the DR-GFP line. The GFP3 filter (Leica Microsystem, Wetzlar, Germany) was mounted on the Leica M165FC fluorescence microscope (Leica Microsystem, Wetzlar, Germany). The genotype of HDR reporter transgenic Daphnia, we co-injected reporter plasmid and the RNP complex into 29 eggs. The 10 injected embryos survived until adult, from which 9 produced offspring with a white eye that is the typical phenotype of the scarlet locus and revealed 20 bp deletion and truncation: a trace of B (ventral), B, and w (dorsal). Strong truncation: only a trace of B and w (dorsal)39.

Quantitative reverse-transcription PCR (qPCR). 
CDnas were synthesized from 1 µg of purified total RNA using random primers and PrimeScript II 1 strand cDNA Synthesis Kit (TaKaRa). The β-actin gene was amplified by PCR to confirm the absence of genomic DNA contamination as previously described33. To quantify repaired SceGFP mRNA level, qPCR was performed in StepOnePlus (Applied Biosystem) using KOD SYBR® qPCR Mix (Toyobo, Osaka, Japan) with primers as listed in Table 2. The cycling condition is as follows: 2 min at 98 °C, followed by a total of 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. Primers’ specificity was confirmed by melting curve analysis and agarose gel electrophoresis. The expression level of the DSB site (Fig. 3B, C, fragment A, DR-GFP line). The 3′ junction region was also amplified in this line by using forward primer targeted at the downstream of EF1α-UTR of the donor plasmid and reverse primer targeted exon 8 of scarlet gene locus (Fig. 3B, C, fragment C, DR-GFP line). Sequencing of this PCR product confirmed the integration of the reporter plasmid at the scarlet locus and revealed 20 bp deletion and 8 bp insertion at the 3′ side of the integrated cassette (Fig. 3D, 3′ junction). Consistent with the white-eyed phenotype, another allele contained indel mutation at the DSB site (Fig. 3D, 2nd allele). We were unable to amplify the 3′ junction region even if the forward primer was designed at 3157 bp upstream and 2610 bp downstream of the DSB site (Fig. 3B, C, fragment A, DR-GFP line). This suggests that large deletion occurred at the 3′ side of the integration site. Nevertheless, amplification and sequencing of the full-length of the DR-GFP gene cassette demonstrated the integration of the intact DR-GFP reporter (Supplementary Figure S1).

| Purpose | Target region | Direction | Sequence |
|---------|---------------|-----------|----------|
| Genotyping | mCherry | Forward | 5′-GGCCATCATCAAGGAGTTC-3′ |
| | 5′ junction region of integration site | Forward | 5′-TGGAAGACGTCATTGATTGC-3′ |
| | Reverse | 5′-CTGGAGTAATAGCGAAAG-3′ |
| | 3′ junction region of integration site | Forward | 5′-CAGCCATAACCATTTTAG-3′ |
| | Reverse | 5′-GTTGAGGACTGTTATCTTC-3′ |
| Repaired eGFP | Forward | 5′-CCAGACACCGCAAAGCTGAAATGACC-3′ |
| | Reverse | 5′-ATGGCCCTTGGCCCTCGCGG-3′ |
| Repaired eGFP | Forward | 5′-TTCATAACATCGGGGATGCTG-3′ |
| qPCR | mCherry | Reverse | 5′-GTCAGGAAGCTGGAATCAGGC-3′ |
| | Reverse | 5′-GGTGATACTCTCCTGTGG-3′ |
| | L32 | Forward | 5′-GACCAATGGTATTGACACAAG-3′ |
| | Reverse | 5′-CCAATTTGTGCAATAGTGT-3′ |

Table 2. List of primers. The primers were synthesized by FASMAC (Tokyo, Japan).

B and P. Mild truncation exhibit, a part of M and full B (ventral ramus), full M, B, and w (dorsal ramus). Medium truncation: a trace of B (ventral), B, and w (dorsal). Strong truncation: only a trace of B and w (dorsal)39.
Figure 3. The phenotype and genotype of the DR-GFP line (A) Comparing phenotypes of the DR-GFP line with the wild type Daphnia. The top and lower rows show D. magna obtained from wild type and DR-GFP lines respectively. The image in each column was photographed using either of bright field, mCherry, or GFP3 filter. The region inside the white-dashed line is the gut, in which ingested chlorella emits slight red autofluorescence both in wild type and DR-GFP. Widespread mCherry fluorescence was observed only in the transgenic line, while eGFP fluorescence was not observed. Black arrow indicates loss of black eye pigment due to disrupted scarlet allele. (B) Schematic representation of the integration site of the DR-GFP donor plasmid. A part of the RHOGAP (Rho-GTPase activating protein) gene is shown upstream of scarlet. The DR-GFP donor plasmid was integrated into exon 3 of the scarlet gene. A and C indicate the 5′ and 3′ junction regions of donor plasmid and genome, B indicated the internal region of donor plasmid (mCherry gene). (C) PCR result was visualized by gel electrophoresis. The first lane is the marker, followed by fragments A, B, C, and β-actin for both wild type and DR-GFP line Daphnia. In the sample using DR-GFP line Daphnia, all fragments except the 5′ junctions were amplified. The full length of the gel is presented in Supplementary Figure S4 (D) 20 bp deletion and 8 bp insertion were detected in the 3′ junction of plasmid integration and another allele of scarlet respectively. The red-colored nucleotides and black triangle indicated the St gRNA target and DSB site respectively.
The DSB near the I-SceI site leads to the generation of eGFP-positive cells in embryos of the DR-GFP line. To examine whether the DR-GFP reporter gene is functional in the established line, we attempted to introduce the DSB near the I-SceI site. Seventy-four eggs were co-injected with 1 µM Cas9 protein and gRNA mixtures (Sce gRNA and Dll gRNA, 2 µM each). Dll gRNA was used as a marker for Cas9 activity during microinjection as described in Materials and methods and Supplementary Table S1. Forty-three embryos survived until the 48 hpi stage and 41 (95%) showed truncation of the second antennae (Table 3) from which, 22 embryos (54%) showed the strong phenotype29, indicating that Cas9 was active during injection and could introduce DSBs on the genome. Of the 41, 33 (80%) showed strong nuclear-localized eGFP fluorescence in the tissues such as the head and thoracic appendages (Fig. 4). In contrast, embryos injected with Cas9 RNP including the unrelated St gRNA (Fig. 4) and Dll gRNA did not show intense and nuclear-localized GFP signal, indicating that the recovery of the eGFP fluorescence occurred by injection of Cas9 protein and Sce gRNA.

The embryos showing the nuclear-localized fluorescence signals have a functional eGFP gene repaired by HDR. To confirm whether HDR occurred at the genomic level, we extracted genome DNA from uninjected embryos and injected embryos that showed nuclear-localized eGFP fluorescence. PCR was then performed with a forward primer in the mCherry region and a reverse primer that recognizes specifically the SceGFP sequence (Fig. 6A). As a model to test this system, we used Cas9-mRNA and Cas9 protein for introducing the DSB on the SceGFP because mutagenesis efficiency with Cas9 mRNA was lower than that with Cas9 protein17, which suggested Cas9 mRNA induces DSB occurrence to a lesser extent. We introduced the DSB at the SceGFP following either optimum condition of Cas9 mRNA (500 ng/µL Cas9 mRNA and 50 ng/µL gRNA) or Cas9 protein injection (1 µM Cas9 protein and 2 µM gRNA). The Dll gRNA was co-injected to evaluate the Cas9 activity in each injection. We confirmed 54% of Cas9 protein injected embryos showed a strong phenotype of second antennae truncation while Cas9 mRNA could only introduce a mild phenotype (Tables 3 and 4). This result implied that Cas9 protein had stronger activity to introduce DSB. Subsequently, the level of repaired SceGFP was analyzed using qPCR. By Cas9 protein injection, we observed significantly higher expression of repaired SceGFP (~fivefold) relative to Cas9 mRNA injection. Moreover, neither repaired SceGFP signal nor amplification was detected in uninjected embryos as well as scarlet gRNA injected embryos (Fig. 6B, Supplementary Figure S3). Our result shows that qPCR can be used to detect the functional eGFP repaired by HDR.

Discussion
Here, we successfully integrated the DR-GFP system into D. magna genome and visualized HDR occurrence in vivo. We evaluated the functionality of this reporter system by introducing targeted DSB in the reporter site. We observed the eGFP signal and detected PCR products from the repaired eGFP gene in the injected daphnids, demonstrating evidence of detection of HDR in situ based on the eGFP fluorescence. We could also detect the repaired eGFP by qPCR that is potentially used for quantitative measurement of the HDR level following DNA DSB occurrences in the future. Furthermore, ubiquitous expression of mCherry that is bicistronically expressed in D. magna could be a promising tool for the evaluation of new genome editing tools (Fig. 1, genome editors).

The DR-GFP system also has been applied for the screening of genotoxicants such as heavy metals37, FDA-approved drugs for cancer therapy38 in addition to evaluation of the sensitivity of the cancer cell to gamma-ray irradiation39. The aquatic ecosystem is constantly exposed to genotoxicants, and D. magna has been long used as a workhorse for ecotoxicology analysis as a biosensor. As the test guideline for acute or chronic toxicity test

| Injected | Developed (48hpi) | Truncated antennae | Nuclear-localized eGFP |
|---------|------------------|--------------------|-----------------------|
| 74      | 43               | Strong: 22/41 (54%) | 33/41 (80%)           |

Table 3. Summary of Cas9 protein, SceI gRNA, and Dll gRNA co-injection.
Figure 4. Detection of eGFP-positive cells of DR-GFP transgenic *D. magna* following the DSB of the SceGFP. DR-GFP line was co-injected with Cas9 protein, SceI gRNA, and Dll gRNA. The injection of Cas9 protein with St gRNA and Dll gRNA was performed as a control. The first row shows uninjected control, while the second, third, and fourth rows show injected individually *Daphnia*. Images were taken using the bright field and the GFP3 filter. All daphniids were photographed at 48 h post-injection. The red dashed line shows the second antennae region, which was truncated because of Dll gRNA injection. The weak background green fluorescence observed throughout the body of all samples was coming from autofluorescence. The repaired SceGFP was controlled under *EF1α-1* promoter/enhancer and contains NLS, resulting in abundantly expressed and nuclear-localized eGFP expression (yellow triangles).
for *Daphnia* is well established by following the OECD Guideline40,41 we believe our DR-GFP may be suitable for screening genotoxicants in vivo (Fig. 1, genotoxicants).

In recent years, several transgenic animals containing the DR-GFP system have already been established for analyzing the molecular mechanisms of HDR. For instance the generation of DR-GFP reporter mouse for analyzing the HDR frequencies in primary cell types derived from diverse lineages21. In *C. elegans*22, the DR-GFP system was integrated into the genome to identify a novel role of protein for promoting HDR. *Daphnia* might have a unique DNA repair mechanism to neutralize the genetic drawbacks because its asexual ability might lead to the accumulation of deleterious mutations due to the absence of recombination events via mating. We anticipate the prospect of utilizing this transgenic *Daphnia* for studying the function and roles of HDR in asexual reproduction.
by manipulating the components of HDR machinery. The result would contribute to a further understanding of evolutionary genomics (Fig. 1, manipulated components).

The HDR efficiency reported in mammals and plants is lower compared to NHEJ because it takes a longer time to complete than NHEJ and functions only during S and G2 phases when the sister chromatid, the main template to repair DSB, is present. Thus, several approaches have been developed to enhance genome editing by HDR such as inhibiting or knocking out the key factor of NHEJ, synchronizing and capturing cells at the certain phases, and modifying the Cas9 by fusing it with a key protein necessary in the HDR steps. To evaluate the effects of these approaches on the HDR activity, the reporter system for visualizing the HDR event has been used in mammalian cells. We suggest the potential use of DR-GFP for optimizing HDR efficiency for instance by impairing the NHEJ repair genes (Ku70 or Lig4) (Fig. 1. manipulated components).

We also acknowledge the limitations of this reporter system. First, in live imaging, it may be difficult to detect the eGFP signals from mutated cells that are located deep inside the tissues, which may lower sensitivity for detecting eGFP positive cells and their quantification. This limitation could be addressed by sorting and counting the eGFP-positive cells using fluorescence-activated cell sorting (FACS). Second, the DR-GFP reporter system can only visualize the presence of HDR events at the reporter locus. This situation may affect the sensitivity for detection of the HDR triggered by environmental genotoxicants or mutagenic agents that may introduce random DSB throughout the genome. Therefore, other approaches to globally visualize HDR events in Daphnia may be developed. For instance, fusing the Förster resonance energy transfer (FRET) system in HDR key proteins to provide spatiotemporal visualization of their function. Third, for a comprehensive understanding of the DNA repair mechanism in this species, reporters for detection of the other DNA repair pathways such as NHEJ and SSA need to be developed. This limitation can be addressed by utilizing other reporter systems, such as “traffic light”, a dual fluorescence-based reporter which can visualize HDR and NHEJ repair pathways. However, despite these possible limitations, DR-GFP reporter Daphnia would offer a valuable tool for the evaluation of HDR in this ecologically important species.

Table 4. Summary of Cas9 mRNA, Scel gRNA, and Dll gRNA co-injection. *To confirm the integrity of the Cas9 mRNA, the eGFP mRNA was also co-injected for confirmation of the mRNA integrity based on the eGFP fluorescence intensity. This prevented us from observing the nuclear-localized eGFP signals in the Cas9 mRNA-injected embryos.

| Injected | Developed (48hpi) | Truncated antennae | Nuclear-localized eGFP |
|----------|------------------|--------------------|------------------------|
|          |                  | Strong | Medium | Mild |                     |
| 24       | 10               | 0      | 0      | 8/8 (100%) | Not observed* |

Figure 6. Detection of the functional eGFP transcript by qPCR. (A) The position of primers and the region used for quantifying the repaired SceGFP level (above) were shown in the black line. The alignment showed that the reverse primer was designed to specifically bind to repaired SceGFP fragment (underline). (B) Level of repaired SceGFP between injected and uninjected samples after the introduction of DSB. The value was quantified by qPCR. There was a significant difference between Cas9 protein and mRNA injection. The values are means and error bars represent standard error (N = 3). *p < 0.05 (Student’s t-test). In uninjected embryos and ones injected with Cas9 protein and scarlet gRNA, the repaired SceGFP mRNA was not detected (ND).
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**Author contributions**

R.M.F., N.A., Y.K., and H.W. conceived and designed the study. R.M.F. made the transgenic animal, performed the main experiments, and wrote the original draft. R.M.F., N.A., and Y.K. edited the manuscript. H.W. supervised the experiments and finalized the manuscript. All authors discussed the results, commented, and reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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