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Spatiotemporal control of transgene expression using an infrared laser in the crustacean *Daphnia magna*

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The crustacean *Daphnia magna* is an emerging model for ecological and toxicological genomics. However, the lack of methods for spatial and temporal control of gene expression has impaired the elucidation of molecular mechanisms underlying responses to environments in vivo. Here we report local activation of the *hsp70* promoter-driven gene cassette in *D. magna* by the infrared laser-evoked gene operator (IR-LEGO), a method for heating the target cells with infrared irradiation. We identified the heat-inducible promoter upstream of the *D. magna hsp70-A* gene. Using this promoter, we generated a transgenic *Daphnia* harboring the heat-shock responsive *GFP* reporter gene and confirmed that the *GFP* gene responds to heat treatment not only in juveniles and adults but also in embryos. We collected embryos from the reporter line and irradiated four different regions of interest in the embryos: a proximal region of the third thoracic segment, a part of the midline, a second maxilla, and a distal region of the endopodite of the second antenna, all of which increased GFP fluorescence with an infrared laser. Our results suggest that the IR-LEGO method is useful for spatial and temporal control of gene expression and would advance the functional genomics in *D. magna*.

Daphnia magna is a small planktonic crustacean that is ubiquitously found in freshwater habitats in Europe, Africa, Asia, and America[1]. It occupies an important position in a freshwater food chain where it is an herbivore consuming algae and a prey for aquatic organisms such as fish. This characteristic has attracted ecologists and environmental scientists for decades, which has led to findings of diverse phenotypic plasticity in response to environmental changes[2]. *D. magna* has also been used for toxicological research because it shows high sensitivity to anthropogenic chemicals[3]. To understand the phenotypic plasticity and response to toxic chemicals at a genetic level, its EST and genome sequence have been determined[4–7]. To analyze the function of genes in *D. magna*, genome editing tools have been developed using CRISPR/Cas9[8,9] and TALEN systems[10–13], which achieved a transgenerational change of gene activity. In addition, during embryogenesis, transient knockdown and overexpression of a target gene are also possible by injecting nucleic acids such as double-stranded RNA[14], mRNA[15], and plasmid DNA[16] into the eggs. However, in these gene manipulation methods, temporal control of gene expression in specific types of cells and tissue is difficult, which has prevented us not only from modulating gene activity in a region of interest of the body but also from labeling and tracing of target cells and tissues at desired developmental stages.

The infrared laser-evoked gene operator (IR-LEGO) is a method to induce local expression of the chromosomally integrated gene cassette by heating the target cells with irradiation using an infrared (IR) laser[17]. This method is based on the induction of heat shock proteins (HSPs) in response to heat treatment in eukaryotes[18]. The promoter region of the *hsp70* gene commonly has nucleotide sequences called heat shock response elements (HSEs) that are bound to the heat shock transcription factor (HSF)[19]. Via binding to the HSEs, HSF activates expression of the *hsp70* gene after heat shock. The IR-LEGO method has been applied to various organisms from plants to animals[20–23]. In this study, we aimed to establish a transgenic *D. magna* harboring the

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heat-inducible GFP reporter and to examine the applicability of the IR-LEGO method to *D. magna* using the established reporter line.

Results

Heat treatment upregulated *Dmhsp70-A* expression

We attempted to find an ortholog of the *hsp70* gene that responds to heat shock in *D. magna*. We first searched a *D. magna* reference genome (GenBank accession no. GCF_020631705.1) for sequences orthologous to *hsp70*. In addition to *Dmhsp70-A* and *Dmhsp70-B* that had been already identified[24], we found the other three *hsp70* orthologs, *Dmhsp70-C*, *Dmhsp70-D*, and *Dmhsp70-E* (Supplementary Table S1). Phylogenetic analysis indicated that *Dmhsp70-A*, *Dmhsp70-B*, and *Dmhsp70-C* are cytosolic whereas *Dmhsp70-D* and *Dmhsp70-E* are endoplasmic reticulum-associated and mitochondrial *hsp70*s respectively (Supplementary Figure S1). Since a cytosolic *hsp70* gene has been known to be upregulated by heat stress, we searched for the putative HSE in the 1,000-bp of each cytosolic *hsp70* promoter. We identified seven HSEs in the *Dmhsp70-A* promoter in contrast to no HSE in the *Dmhsp70-B* and *Dmhsp70-C* promoters (Supplementary Figure S2, Supplementary Table S2). These sequence analyses prompted us to investigate the heat shock response of *Dmhsp70-A*.

To determine the temperature and time for inducing heat shock response, we investigated how acute thermal stress affected the survival rate of two weeks old *D. magna*. We conferred heat shock to the adult daphniids at 34, 36, 38, and 40 °C for 90 min and, during this heat treatment, we examined temporal changes of the survival rate at each temperature. Although no daphniid survived for 25 min at 38 and 40 °C, the other treatments at 34 and 36 °C did not reduce the survival rate for at least 90 and 60 min respectively (Supplementary Figure S3). Based on these acute thermal responses, to investigate the response of the *Dmhsp70-A* gene to thermal treatment, we chose heat shock at 36 °C for 15 min or 30 min. We extracted total RNAs from the treated animals, synthesized their cDNAs, and analyzed gene expression changes using the qPCR. The heat exposure induced *Dmhsp70-A* gene activation (Fig. 1). These results revealed that heat-shock treatment activates the *Dmhsp70-A* gene.

Integration of the *Dmhsp70-A* promoter-driven GFP reporter gene into the genome

We amplified the *Dmhsp70-A* DNA fragment that is composed of the 359-bp promoter region harboring seven HSEs and 5′ UTR by genomic PCR. We previously had constructed the metal inducible GFP reporter plasmid that is controlled by the promoter and 5′ UTR of the *metallothionein A* (*MT-A*) gene[25]. We replaced the *MT-A* gene regulatory region of this metal reporter plasmid with the obtained genomic DNA fragment from *Dmhsp70-A* (Supplementary Figure S4). Since we had reported that the *Daphnia magna scarlet* gene can be used as a target site for gene integration[26–28], we included a recognition sequence of *scarlet*-targeting gRNA to the constructed plasmid, which resulted in the generation of the donor plasmid for Cas9-mediated non-homologous end-joining repair (Supplementary Figure S2). We co-injected the donor plasmid with Cas9 ribonucleoproteins including the *scarlet*-targeting gRNA into 24 eggs. Of these injected eggs, 20 and 13 survived until juvenile and adult stages respectively. One founder animal produced offspring showing GFP fluorescence. Transmission of the GFP reporter gene was confirmed by genomic PCR (Supplementary Figures S5 and S6). We also sequenced the 5′ and 3′ junctions (Supplementary Figure S7), revealing the integration of the donor plasmid into the target site. Taken together, we could obtain one transgenic line designated as *D. magna* HSE-GFP. The HSE-GFP line has no apparent change in growth and reproduction (Supplementary Figure S8), which led to the maintenance of this line under the conventional culturing condition in the laboratory.

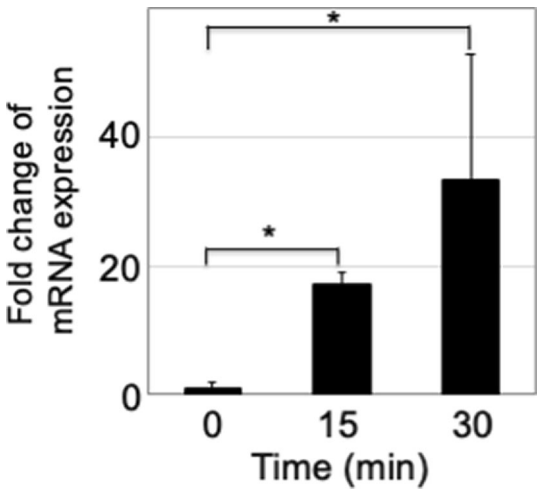

Fig. 1. Activation of*Dmhsp70-A* expression after heat exposure. The expression level before heat exposure (0 min) was designated as one and the magnitude of changes in expression was indicated for each time point. Error bars indicate the standard error of the mean ($n = 3$). $*p < 0.05$ (Student's t-test).

The chromosomally integrated GFP reporter gene responds to heat exposure

To investigate whether heat exposure increases the GFP expression of the genetically encoded reporter of the HSE-GFP line, we incubated daphniids at 36 °C for 5 min during any of three different developmental stages (embryo, juvenile, and adult) and observed GFP fluorescence at 24 h after the heat exposure (Fig. 2). Under conventional culturing conditions at 23 ± 1 °C, in the wild-type, green autofluorescence is not detected[16]. First, we collected embryos around stage 4[29] and exposed them to heat stress. Without heat exposure, embryos showed ubiquitous and weak GFP fluorescence. The reporter GFP gene responded to heat stress and the treated embryos exhibited more uniform and stronger fluorescence than non-treated embryos (Fig. 2, embryo). Second, juveniles less than 24 h old were collected and exposed to heat stress. The exposed juveniles increased GFP expression ubiquitously and among organs, the gut showed the highest GFP fluorescence (Fig. 2, juvenile). Third, we exposed 10-day-old adults to heat stress. Similar to juveniles, heat treatment led to ubiquitous GFP expression. However, rather than being concentrated in the gut, the GFP signals were stronger in cells that are broadly distributed along the gut and potentially equivalent to fat cells (Fig. 2, adult). Juveniles and adults also had weaker GFP fluorescence without heat exposure. These results suggest that the chromosomally integrated GFP reporter gene responds to heat exposure throughout the life span.

Local activation of the GFP reporter gene by IR laser irradiation in embryos

We examined the effect of IR laser irradiation for temporal and spatial activation of the GFP reporter gene in embryos of the HSE-GFP line. Previous studies reported that an IR-LEGO system[17] can be used for activation of the heat-inducible promoter-driven gene in cells and tissues irradiated with the IR laser[21–23]. For irradiation, we chose the embryos at approximately stage 7.5 that had both naupliar and postnaupliar segments[29] (Fig. 3a), which allowed us to know where the IR laser was irradiated more easily than embryos at the earlier stages. We put an embryo on the glass with its ventral region touching the glass surface and irradiated it with IR from the underside of the glass. For testing the availability of IR-LEGO on gene manipulation of *D. magna*, we needed to avoid variations of water volume between the target tissues and glass as much as possible among experiments because water declines the power of the infrared laser. Therefore, we have chosen four different regions of interest in the embryos: a proximal region of the third thoracic segment, a part of the midline, a second maxilla, and a distal region of the endopodite of the second antenna, all of which could be stably close to the glass surface so that there was little space for water between each body part and the glass. To determine an optimal laser power for inducing heat shock-dependent gene activation, we irradiated the proximal region of the third thoracic segment (t3) with the IR laser using 40x objective and a power range of 16–20 mW for 1 s (Fig. 3a, an asterisk in the t3 region). Irradiation with an IR laser using a power of 20 mW increased GFP fluorescence at the target region in all of the treated embryos and the GFP induction efficiency was reduced when the laser power became weaker (Table 1; Fig. 3b), which prompted us to apply the IR-LEGO system with a power of 20 mW to the three other tissues of the embryos, midline, second maxilla, and second antenna. We irradiated a part of the midline adjacent to the second thoracic segment (t2) (Fig. 3a, an asterisk in the midline region) and observed stronger GFP fluorescence exclusively in this targeted region (Fig. 3c). The second maxilla and a distal region of the endopodite of the second antenna were also irradiated by the IR laser (Supplementary Figure S6a and S6d). In both tissues, only the irradiated part showed an increase in GFP fluorescence (Supplementary Figure S9). Regardless of the irradiated tissues, the induction of GFP fluorescence was detected in each target tissue of all the survived embryos (Table 1), indicating that the IR-LEGO system can induce local activation of the target gene under the *Dmhsp70-A* promoter in *D. magna* embryos.

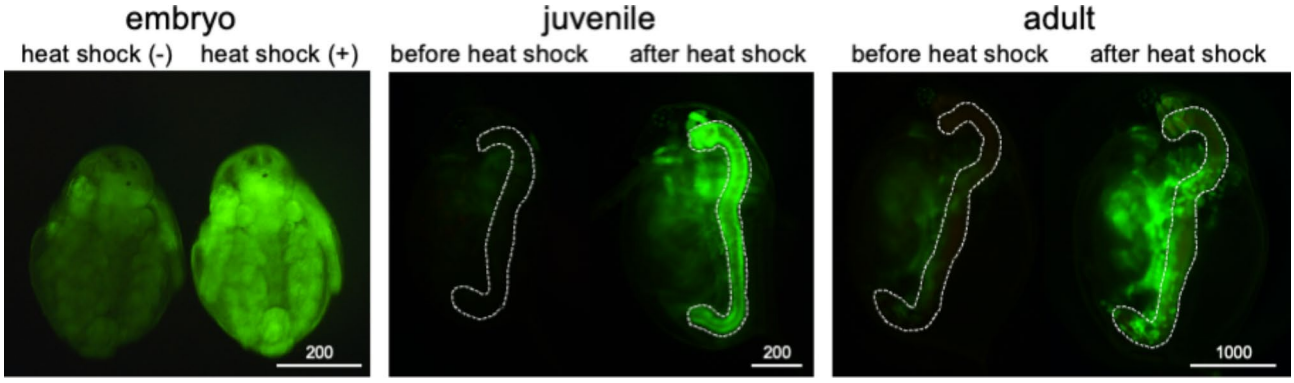

Fig. 2. Induction of GFP expression of heat-exposed*D. magna* HSE-GFP line. Daphniids at three different stages (embryo, juvenile, and adult) were exposed to thermal stress at 36 °C for 5 min and cultured 24 h after heat shock. Because, during embryonic stages, morphological changes were drastic, the fluorescence of the heat-exposed embryo was compared with that of the non-treated embryo at the same stage. At the juvenile and adult stages, left and right pictures indicated daphniids before and after heat shock respectively. The regions surrounded by the dotted lines indicate guts. Each picture was taken with different settings. Bars indicate length in micrometers (μm).

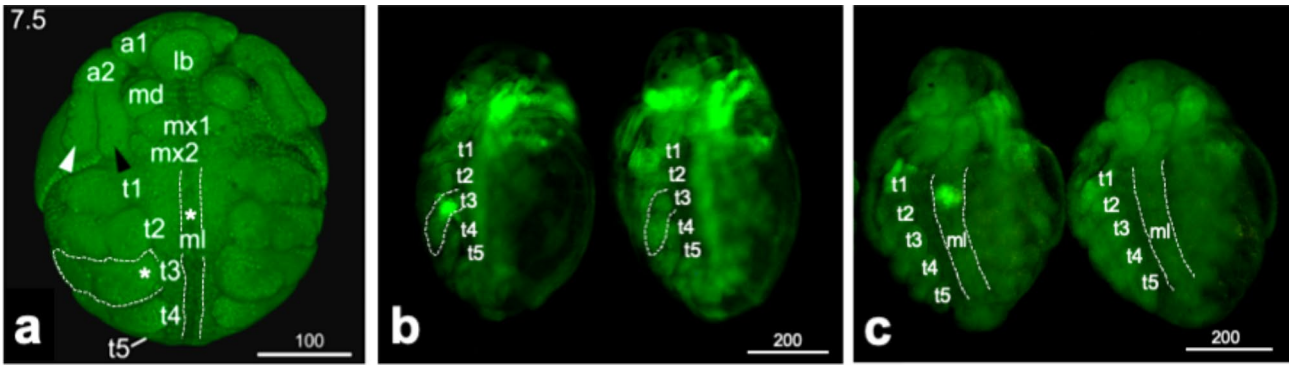

Fig. 3. IR laser-induced GFP expression in the third thoracic segment and midline of*D. magna* embryos. (**a**) A ventral view of an embryo at stage 7.5. An IR laser was irradiated to embryos around at this stage. The location of the irradiation was shown with asterisks. The picture was adapted and modified from Mittmann et al.[29]. The treated embryos were cultured at 15 °C for around 20 h. The black arrowhead indicates the endopodite; the white arrowhead indicates the exopodite of the second antennae. (**b**) A ventrolateral view of embryos with (left) and without (right) irradiation to the proximal region of the third thoracic segment (t3). (**c**) A ventral view of embryos with (left) and without (right) irradiation to the midline. Each picture was taken with different settings. Bars indicate length in micrometers (μm). a1, a2 = first and second antennae; lb = labrum; md = mandible; ml = midline; mx1, mx2 = first and second maxillae; t = thoracic segment.

	Laser power	Irradiated	Survived	Local fluorescence
Third thoracic segment (t3)	16 mW	10	9	0
	18 mW	9	7	2
	20 mW	8	7	7
Midline (ml)	20 mW	6	3	3
Second maxilla (mx2)	20 mW	6	6	6
Second antenna (a2)	20 mW	9	7	7

Table 1. Summary of the IR-LEGO experiments.

Discussion

Here we report the spatio-temporal regulation of the target gene expression using the heat-inducible promoter in the crustacean *Daphnia magna*. Temporal regulation was done by changing the timing of heat exposure from embryos to adults. Spatial regulation was achieved in embryos by irradiation of the target tissues or cells with an IR laser using the IR-LEGO system. The irradiated cells increased GFP fluorescence significantly more than the other cells, which suggests that the combination of the HSE-GFP line with the IR-LEGO system can be used for labeling and tracing target cells and tissues. This local gene activation method could be applied to different developmental stages such as juveniles and adults in the future.

We found GFP background fluorescence without heat treatment in the HSE-GFP line. Lowering the temperature from 23 °C to 15 °C did not reduce the GFP fluorescence (Supplementary Figure S10), suggesting that the heat-inducible promoter is leaky. We will need to overcome the leakiness of the heat-inducible promoter for more precise control of genes of interest in *D. magna*. Leakiness of *hsp70* promoter has been reported in various organisms despite its common use for gene activation[30,31]. For reducing the leaky expression, the artificial promoter containing multimerized HSEs and a minimal promoter has been used for the heat-dependent Cre expression in Medaka fish[23,32–34]. With an IR laser, the expressed Cre proteins led to the excision of elements flanking two loxP sites, resulting in the GFP expression for permanent labeling of the irradiated cells[23]. Dual control of gene expression by the heat-inducible system and the other regulatory system also may overcome the leaky expression derived from the *hsp70* promoter as reported in Zebrafish[31] and *Drosophila*[30]. In zebrafish, the heat-inducible system was combined with the hormone-dependent regulatory system for the activation of the Cre recombinase gene. The Cre recombinase was fused with the estrogen receptor, which led to nuclear translocation of the heat-inducible Cre in an estrogen-dependent manner[31]. In *Drosophila*, to reduce unintended expression from the heat-inducible system, repressive elements like the polycomb response elements were added adjacent to the HSE[30]. Further improvement of the *D. magna* heat-responsive reporter gene will enable us to modulate gene activity in a region of interest of the body more stringently.

The IR-LEGO system may become an important tool for investigating functions of ecologically relevant genes at a cellular level in *Daphnia*. This organism is a keystone species in the freshwater ecosystem and adapts to the surrounding environment in response to abiotic and biotic factors, which contributes to the maintenance of the ecosystem as a primary consumer. Environmental cues are detected by the nervous systems and often converted into hormonal signaling via neuroendocrine cells. In the case of environmental sex determination in

D. magna, the sesquiterpenoid hormones influence oocytes, resulting in the commitment of sex of the offspring to males[35,36]. During differentiation into males, the transcription factor Vrille is transiently activated only during the gastrulation stage and possibly in a limited number of cells for activation of the male-determining gene *Doublesex1* (*Dsx1*)[37]. *Dsx1* is expressed exclusively in male-specific traits[12,38]. Not only in environmental sex determination but also in the other phenotypic plasticity and stress responses such as the predator defense in *Daphnia pulex*[39,40], perception of environmental signals and the resulting signaling cascades would occur locally and transiently. Thus, the spatiotemporal manipulation of genes would be indispensable for understanding how these stage-, cell-, and tissue-specific environmental responses occur, which in turn provides us a deeper molecular insight into the environmental adaptation of *Daphnia* in an ecological context.

In summary, we established a transgenic *D. magna* that responds to heat shock and exhibits GFP fluorescence. Using this transgenic animal, we demonstrated that the IR-LEGO system is useful for spatial and temporal control of gene manipulation in *D. magna*. We anticipate that this system leads to elucidating molecular mechanisms underlying responses of *D. magna* to environments in vivo.

Materials and methods

D. magna 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 multiple generations. Aachener Daphnien Medium (ADaM) was used as the culturing medium. Eighty juveniles (less than 24 h old) were cultured in 5 L of ADaM and fed daily with 80 µL of 7.0×10^9 cells/mL of the green alga *Chlorella vulgaris* and 20 µL of a 0.15 g/mL of baker's yeast (Marsan Pantry, Ehime, Japan). All daphniids were cultured at 23 ± 1 °C, under a constant light/dark photoperiod of 16 h/8 h.

Whole-body heat treatment of *D. magna*

For heat exposure to juveniles and adults, the ADaM in a 50 ml conical tube was incubated to reach appropriate temperatures using a plastic water bath SM-05R (TAITEC, Saitama, Japan). The stirring method in the bath is jet flow and its temperature control accuracy is ±0.1 °C. Four juveniles or adults were put in the pre-warmed ADaM and incubated for heat shock. For heat exposure to the embryos, a glass petri dish containing the ADaM was placed on a rack inside the empty water bath. The water bath was filled with tap water until the water level reached approximately half the height of the petri dish for heating the ADaM to 36 °C. Embryos were put into the pre-warmed ADaM in the petri dish. After the heat exposure, treated individuals were cultured in the medium at 23 ± 1 °C.

Bioinformatics analysis of *D. magna hsp70* genes

The genomic locations of orthologs of *hsp70* were investigated by tBLASTn searches against the genome of the *D. magna* NIES strain (Genome assembly ASM2063170v1.1). The amino acid sequence of *D. magna* heat shock 70 kDa protein cognate 4 (Accession Number: XP_032781380.2) was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) and used as a query. The promoter region of the identified gene from −1,000 to +1 bp was searched for the candidates of the HSE with the matrix profile MA0486.2 in the JASPAR database and RSAT 2022: regulatory sequence analysis tools[41]. HSP70 and the other HSP amino acid sequences from several organisms were obtained from the database (Supplementary Table S3). A multiple alignment was constructed using Clustal W with the following settings: pairwise alignment parameters: gap opening penalty 10.00, gap extension penalty 0.10, identity protein weight matrix; multiple alignment parameters: gap opening penalty 10.00, gap extension penalty 0.20, delay divergent cutoff 30%. The phylogenetic tree was then constructed using the p-distance algorithm and the neighbor-joining method implemented in MEGA version 11[42].

Quantification of mRNA

Total RNA was extracted from daphniids using Sepasol-RNA I Super G solution (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Concentrations of RNA were determined by A260 measurement with Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA solution was treated with DNase I, Amplification Grade (Invitrogen, Carlsbad, CA, USA) for 10 min at 65 °C. Around 2 µg of total RNA was used in cDNA synthesis with random primers (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed with SYBR GreenER qPCR Supermix Universal (Invitrogen) using the Mx3005P real-time (RT)-PCR system (Agilent Technologies, Santa Clara, CA, USA). PCR amplifications were performed with 200 nM primers in triplicate using the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C) in a volume of 20 µl. The primers for the *Dmhsp70-A* gene quantification were HSP-A right: 5′-AAGACGACAAGGTCAAAGACAAGA-3′; HSP-A left: 5′-ACCACTTAATGGCTTCACTGCAC-3′. Gel electrophoresis and melting curve analyses were performed to confirm the correct amplicon size and the absence of the nonspecific band. The target mRNA level was normalized to the transcript level of ribosomal protein L32 [43]. For the estimation of the gene expression levels, the $2^{-\Delta\Delta C_T}$ method was used. Three biological replicates were used in this experiment.

Generation of transgenic *Daphnia*

To make a reporter gene that responds to heat shock, the promoter region of *Dmhsp70-A* was fused to the *GFP* gene. The *Dmhsp70-A* DNA fragment that is composed of the 359-bp promoter region harboring seven HSEs and 5′ UTR was amplified from purified genomic DNA by PCR. The primers for amplification of the *Dmhsp70-A* DNA fragment were HSP-A HSE right: 5′-TGCCATGCTGGAATACACCTAC-3′; HSP-A HSE left: 5′- CA CGACTGATCAACGTCCTTCTAC-3′. *Daphnia* genomic DNA was prepared as described previously[16]. The

amplified DNA fragment was cloned into a pCR-Blunt II-TOPO vector (Thermo Fisher Scientific), generating pCR-HSP70-A. After the confirmation of the sequence, the *Dmhsp70-A* promoter region and 5′ UTR were amplified using primers 5′-GCAGAATTCGCCCTTAAATTCATATAGCATTACTTAGG-3′ and 5′-GCCCTT GCTCACCATTTCTGTTGATTAAACAAAC-3′. The *AcGFP1* coding sequence, SV40 poly-A signal sequence, and vector sequence were also amplified from pCR-MTApro-GFP[25] using primers 5′-AAGGGCGAATTCTG CAGATATCCATCAC-3′ and 5′-ATGGTGAGCAAGGGCGCCGA-3′. By fusing these two DNA fragments using the In-fusion HD Cloning Kit (Clontech Laboratories, Inc., CA, USA), the *AcGFP1* coding sequence with the poly-A signal sequence was inserted under the control of *Dmhsp70-A* promoter, which was designated as pCR-HSP70Apro-GFP (Supplementary Figure S4). For its integration into the *D. magna* genome, a part of the *scarlet* gene harboring a guide RNA (gRNA) target sequence[26–28] was added to the plasmid. The nucleotide sequence of the heat shock reporter is provided in Supplementary Figure S2. For microinjection, the reporter plasmid concentration was adjusted to 50 ng/μL.

Microinjection was performed following an established protocol using the *S. pyogenes*-originated Cas9 proteins[8,14]. The Cas9 proteins were expressed in *E. coli* strain BL21 (DE3) and purified following established protocol[44]. Cas9 protein and *scarlet* targeting gRNA were incubated at 37 °C for 5 min to form a ribonucleoprotein (RNP) complex. Eggs were collected from two- to three-week-old *Daphnia* just after ovulation, placed in ice-cold M4 medium[45] containing 80 mM sucrose, and injected with approximately 0.3 nL of a solution containing the reporter plasmid and Cas9 RNP including 2 μM gRNA and 1 μM Cas9 protein. The injected eggs were incubated in a 96-well chamber at 23 °C until the first juvenile instar stage. The transgenic line was screened by the expression of GFP in offspring. GFP expression was observed under a Leica M165C fluorescence stereoscopic microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) fitted with a 480-nm excitation filter and a 510-nm barrier filter (GFP2 filter set). The bright field and fluorescence images were taken with the DFC500 color camera (Leica Microsystems Heidelberg GmbH).

Integration of the donor DNA into the target site was confirmed by genomic PCR. *Daphnia* genomic DNA was prepared as described previously[16]. Based on the genome sequence of the *D. magna* Xinb3 strain that had been publicly available first among genome sequences of the *D. manga* strains (daphmag2.4, https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_001632505.1/), the primers were designed at 304 bp upstream and 290 bp downstream of the Cas9 cleavage site. In PCR reactions, the designed primers that recognize upstream (5′-TAC AGTACCTCTTGCCGTACTCG-3′) and downstream (5′-TGATAAACGTAGCCGCTC-3′) of the cleavage site were used with the antisense primer (5′-AACTTCAGGGTCAGCTTGCC-3′) and sense primer (5′-CTGCTG CCCGATAACCACTA-3′) in the *AcGFP1*-coding region to amplify the 5′ and 3′ junctions between the donor plasmid and genome respectively.

Irradiation of embryos using the IR-LEGO system

For attempting reduction of background fluorescence, the transgenic daphniids were cultured at 15 °C under a constant light/dark photoperiod of 16 h/8 h. Embryos around stage 7.5[29] were collected from two- to three-week-old adults of the transgenic *Daphnia*. A 500-μm mesh filter separated from a sterile PluriStrainer filter (pluriSelect Life Science, Leipzig, Germany) was physically fixed on a cover glass (24 × 50 mm NEO micro cover glass, Matsunami Ind., Ltd., Osaka, Japan) with the vinyl tape and covered with the M4 medium[45]. The single embryo was put into each mesh hole with its ventral region touching the glass surface. Irradiation using an IR (1,480 nm) laser was performed as described previously[21–23]. An IR-LEGO 1000 system (Sigma-Koki, Saitama, Japan) equipped with an IX81 inverted microscope (Olympus, Tokyo, Japan) and a custom-made 40x objective lens (UAPO340 40x/0.90 UV; Olympus, Tokyo, Japan) was used for laser irradiation. Embryos were irradiated at 16–20 mW for 1 s and then incubated in a 96-well chamber at 15 °C under dark conditions. At around 20 h after the irradiation, GFP expression was observed under a Leica M205FA fluorescence stereomicroscope (Leica Microsystems Heidelberg GmbH) fitted with a 480-nm excitation filter and a 510-nm barrier filter (GFP2 filter set). The fluorescence images were taken with the DFC360FX monochrome camera (Leica Microsystems Heidelberg GmbH).

Data availability

The datasets analysed during the current study are available in the NCBI repository, GCF_020631705.1 to *D. magna* reference genome, GCA_001632505.1/ to D. manga Xinb3 strain genome, and XP_032781380.2 to D. magna heat shock 70 kDa protein cognate 4.

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Author contributions
Yasuhiko Kato and H.W. conceived this study. R.S., N.A., and P.R performed hsp70 promoter analysis, cloning, gene expression analysis, and generation of the HSE-GFP line. J.S., M.F., and Yasuhiko Kato performed the IR-LEGO experiment. Yasuhiro Kamei supervised the IR-LEGO experiment. H.W. supervised the whole project. Yasuhiko Kato wrote the first draft of the manuscript. All authors reviewed and approved the manuscript.

Declarations

Competing interests
The authors declare no competing interests. The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

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