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Original Article

Screening of morphology-related genes based on predator-induced transcriptome sequencing and the functional analysis of *Dagcut* gene in *Daphnia galeata*

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Abstract

High fish predation pressure can trigger "induced defense" in *Daphnia* species, resulting in phenotypic plasticity in morphology, behavior, or life-history traits. The molecular mechanisms of defense morphogenesis (e.g., the tail spine and helmet) in *Daphnia* remain unclear. In the present study, the tail spine, helmet, and body of *Daphnia galeata* under fish and non-fish kairomones conditions were collected for transcriptome analysis. A total of 24 candidate genes related to the morphological defense of *D. galeata* were identified, including 2 trypsin, one cuticle protein, 1 C1qDC protein, and 2 ferritin genes. The function of the *Dagcut* gene (*D. galeata* cuticle protein gene) in relation to tail spine morphology was assessed using RNA interference (RNAi). Compared with the EGFP (Enhanced green fluorescent protein) treatment, after RNAi, the expression levels of the *Dagcut* gene (*D. galeata* cuticle protein gene) showed a significant decrease. Correspondingly, the tail spines of the offspring produced by *D. galeata* after RNAi of the *Dagcut* gene appeared curved during the experiment. In whole-mount in situ hybridization, a clear signal site was detected on the tail spine of *D. galeata* before RNAi which disappeared after RNAi. Our results suggest that the *Dagcut* gene may play an important role in tail spine formation of *D. galeata*, and will provide a theoretical basis for studying the molecular mechanisms of the morphological plasticity in cladocera in the future.

Key words: *Dagcut*, *Daphnia galeata*, fish kairomones, RNAi, tail spine, transcriptome sequencing.

Daphnia are planktonic crustaceans that link nutritional levels between primary producers (phytoplankton) and consumers (e.g., fish and zooplankton) in freshwater ecosystems (Lampert 2011). Inducible defenses in *Daphnia* have been extensively reported in the presence of predators (Gilbert 2004; Miyakawa et al. 2010; Ma et al. 2016). Specifically, predator pheromones can induce changes in the morphological characteristics of *Daphnia*, such as longer tail spines and helmets and the occurrence of neckteeth (Weber and Vesela 2002; Dzialowski et al. 2003; Rabus et al. 2012; Rozenberg et al. 2015; Ma et al. 2016). For example, a wider head and longer helmet in *D. galeata* occurred in the presence of a fish (*Perca*) or *Chaoborus* larvae kairomone (Weber and Vesela 2002). Ma et al. (2016) observed significantly smaller body length and prominent recurvate helmets of *Daphnia sinensis* in several large Chinese lakes where predators were present. *Daphnia magna* also develop a bulky morphotype (longer body length and width and an elongated tail spine) as an effective inducible defense against the predatory *Triops cancriformis* (Rabus et al. 2012, 2013). Moreover, the cuticle of *D. magna* exposed to the predator *T. cancriformis* was harder and thicker than that of the control *Daphnia* (Rabus et al. 2013; Otte et al. 2014), and a significant positive correlation between *Triops* density and the expression of defensive traits in *D. magna* was observed in a long-term mesocosm investigation (Rabus et al. 2012). In addition, chemical cues released from some invertebrate predators (*Chaoborus flavicans*, *Leptodora kindtii* and *Cyclops* sp.) induced significantly longer helmets and tail spines in *Daphnia cucullata* (Laforsch and Tollrian 2004). In *Daphnia lumholtzi*, Dzialowski et al. (2003) observed that neonates produced from mothers exposed to fish (*Lepomis*) kairomones had significantly longer helmets and tail spines than those produced from control and *Chaoborus* kairomones. However, investigations of the molecular mechanisms of these inducible defenses of *Daphnia* species against predators have been limited.

Some investigations have showed that the formation of neckteeth in *Daphnia pulex* is related to genes in endocrine and juvenile hormone pathways (Miyakawa et al. 2010; Weiss et al. 2012; Miyakawa et al. 2013). Christjani et al. (2016) found that the neckteeth induction of *D. pulex* was associated with 2 chitin deacetylase genes. Cuticle proteins and chitin filaments are the primary components of the cuticle (Yang et al. 2020). Furthermore, some cuticle protein genes have been identified in invertebrates (Wu et al. 2016; Shang et al. 2019; Yang et al. 2020). Additionally, using the 2D-DIGE technique, Otte et al. (2014) confirmed that kairomone exposure of *T. cancriformis* increased the

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intensity of spots containing muscle proteins, cuticle proteins and chitin-modifying enzymes as well as enzymes of carbohydrate and energy metabolism in *D. magna*, which indicated the inducible defenses of *D. magna* to the predator. Specifically, in the presence of an invertebrate predator, the expression levels of cuticle genes, zinc-metalloproteinases and vitellogenin genes increased, while the C-type lectins and proteins involved in lipogenesis decreased in *D. pulex*. Some effector genes related to morphological alterations and the expression of proteins involved in formation of protective structures and in cuticle strengthening were identified in *D. pulex* using transcriptome sequencing (RNA-seq) data (Rozenberg et al. 2015). In addition, previous studies showed cuticle protein genes played key roles on molting and seemed no significant correlation with developmental rate in insects (Mun et al. 2015; Wu et al. 2016) and *Daphnia* (Soetaert et al. 2007). Although some morphology-related genes have been identified in crustaceans, the molecular mechanisms of induced morphogenesis (e.g., the helmet and the tail spine) in *Daphnia* species remain unclear.

Currently, transcriptome sequencing (RNA-seq) and RNA interference (RNAi) technology are commonly used to screen candidate genes in *Daphnia* species (Huylmans et al. 2016; Herrmann et al. 2018; Ravindran et al. 2021; Wang et al. 2022), and have revealed the function of candidate genes in cladocera (Kato et al. 2011; Hiruta et al. 2013; Chen et al. 2014; Schumpert et al. 2015; Street et al. 2019). Through ingestion of bacteria expressing dsRNA, RNAi has been employed to exploit the functions of some specific genes in *Daphnia* (Schumpert et al. 2015; Street et al. 2019). In this study, our goals are to screen morphology-related candidate genes by transcriptome sequencing of different body parts from *D. galeata* exposed to fish kairomones (FK). Moreover, through transcriptome sequence of 3 body parts and RT-qPCR analysis, 4 candidate genes were significantly related to the tail spine defense of *D. galeata*, among which a gene (Cluster-2384.10979, *Dagcut*) was annotated as cuticle protein in PFAM database, so we chose the *Dagcut* gene to explore the molecular function in affecting the tail spine of *D. galeata* by RNAi and detect its expression sites by whole in situ hybridization. Our research results revealed for the first time the role of the *Dagcut* gene in influencing the tail spine formation of *D. galeata*, and provide a reliable molecular method for screening and exploring morphology-related genes in cladocera.

Materials and Methods

Collection of fish kairomones and culture of *Tetradesmus obliquus*

To obtain FK, one 1.5-kg *Aristichthys mobilis* was placed in an aquarium containing 30 L aerated tap water for 48 h. After 24 h without food, the tap water was replaced, and *A. mobilis* was cultivated for another 24 h, and the fish-conditioned water was filtered using 0.45 μm Whatman GF/F glass microfibre filters. The filtered fish-conditioned water was then placed in a sealed packet and stored at −20 °C. The FK were prepared according to the method described by Jansen et al. (2013).

Two FK concentration treatments were used: 0% fish-conditioned water + 100% culture medium (control) and 20% fish-conditioned water + 80% culture medium. The culture medium is filtered tap water that has been aerated for more than 48 h.

T. obliquus was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan, China. *T. obliquus* was cultured in BG11 medium at 25 ± 1 °C under an illumination intensity of 2,500 lx, with a 12 h: 12 h light/dark cycle, and then collected at the exponential growth period, and stored at 4 °C.

Collection of *D. galeata* samples for transcriptome analysis

D. galeata were hatched from resting eggs in the sediments of Lake Chaohu and cultured at 25 °C. One clone of *D. galeata* was chosen for this experiment. Ten neonates (birth time < 6 h) in the third generation were cultured in 50 mL beakers with 40 mL of culture medium. *T. obliquus* was used as the food source for *D. galeata,* at a concentration of 2×10^5 cells mL^{-1}. The experiment was conducted in a constant-temperature illumination incubator at 25 ± 1 °C, with a 12,h: 12,h light/dark cycle and an illumination intensity of 2500 lx. The culture medium is filtered tap water that has been aerated for more than 48 h. In the control group experiment, a total of 2,200 neonates were cultured in 220 beakers (10 neonates in each beaker), respectively. Similarly, in the fish kairomone group experiment, a total of other 2,200 neonates were also cultured in 220 beakers, respectively. During the experiment, the culture medium was replaced every day. When these individuals grew to 1 instar before first maturity (the fourth instar), their helmet, tail spine, and body of *D. galeata* were respectively cut (Figure 1). BH, BT, and BR stand for the helmet, tail spine and body in the control group, respectively, whereas PH, PT, and PR stand for the helmet, tail spine and body in the fish kairomone group, respectively. Approximately 1,000 helmets, tail spines and

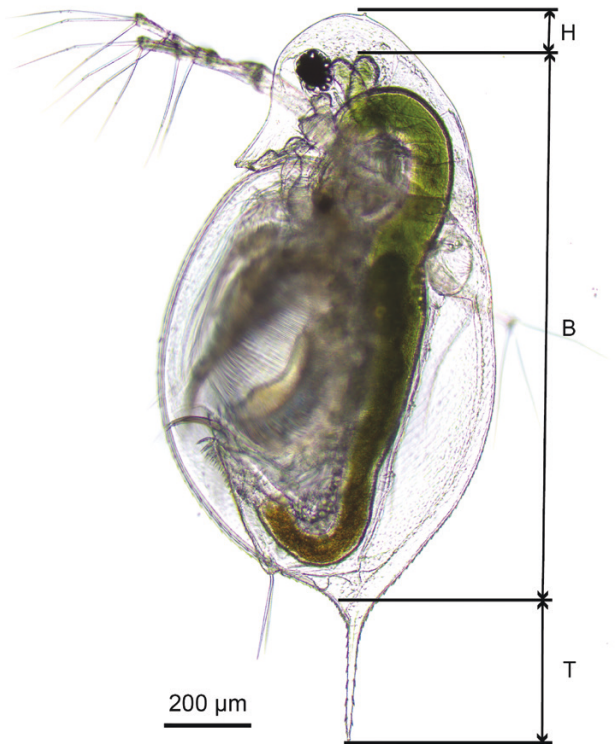

Figure 1 A photograph of *D. galeata*. H: helmet; B: body; T: tail spine.

Table 1 The primer sequence of gene screening in the experiment

Primer name	Primer sequence (5′–3′)
Cluster-2384.9683-F	CACCCAATCAAGGAGACT
Cluster-2384.9683-R	ACACCGATGAATAACCAGTA
Cluster-2384.3055-F	AGCAGCCAACTTCTTACT
Cluster-2384.3055-R	TTAATACGGTCAACGAACAC
Cluster-2384.9311-F	TCAGCATCTACCGTTACC
Cluster-2384.9311-R	TCAGCATCTACCGTTACC
Cluster-2384.2380-F	TGACAACCAAGACTCACA
Cluster-2384.2380-R	TTAGCAATCCAAGGCAATG
Cluster-2384.4388-F	CAACAAGTGCTCAACATCT
Cluster-2384.4388-R	CAACCATCAAGTGCCTTC
Cluster-2384.5440-F	TTCTTCAGCCTTGTTATCCT
Cluster-2384.5440-R	CATCGCAAGTAGTGTAAGTC
Cluster-2384.10979-F	CACCAGCAAACTCTTATTGA
Cluster-2384.10979-R	TGACTAACCTAACGAATGGA
Cluster-2384.16890-F	AAGTGGAGGATATGGCAATA
Cluster-2384.16890-R	AATCTGTTGAGTGGTTGTG
Cluster-2384.10346-F	CTTGATTGTCGCCATCTG
Cluster-2384.10346-R	CTGTTGCTACTTCCATCTTC
Cluster-2384.4417-F	CAGGAGTAGCAGTAGTGTT
Cluster-2384.4417-R	CAGTAGCAGTGTAGGTGTA
Cluster-2384.8245-F	ACCAATCTGTCATCACTGA
Cluster-2384.8245-R	CCGTCTGATAACACTCTTAAC
Cluster-2384.13779-F	TCGCAATGTAAGAGTCAATG
Cluster-2384.13779-R	CAGTTAAGAGGCAAGTTACC
Cluster-2384.11140-F	ACAAGTGAGTGCTCTGAA
Cluster-2384.11140-R	CCGAAGACACGACTGATA
Cluster-2384.12328-F	GAGTCCTTCGTTGATTGC
Cluster-2384.12328-R	GTTGACATTGACGCCTTAA
Cluster-2384.12999-F	CACCCATAGTAGAAGAAACAC
Cluster-2384.12999-R	GAGCCTTGATGAGGATGA
Cluster-2384.8263-F	ATGTGACTGCGTAGATGAT
Cluster-2384.8263-R	CTTGGTGGTGTTGATTGG
Cluster-2384.3878-F	GGACAAGCTAAGTGGACTA
Cluster-2384.3878-R	TCAAGACAGGAAGTTGGTAT
Cluster-2384.6917-F	GGATGACGAAGGACTTGA
Cluster-2384.6917-R	GTTCCACCACACAGACTA
Cluster-2384.7946-F	AGTGCGGTAATCAATGGA
Cluster-2384.7946-R	GGACACAACAAGTTCTCTG
Cluster-2384.9831-F	GCCGTAAAGTCGTATTGATT
Cluster-2384.9831-R	TTGGTATTAGACTCGTCGTT
Cluster-2384.7175-F	ACTACTTCAACAGCAATGC
Cluster-2384.7175-R	CTGGCTACTGGCTACTAAA
Cluster-2384.16699-F	GAGAACTGGTGGTGAGAT
Cluster-2384.16699-R	CAATAGACTTCCGCTTGAAT
Cluster-2384.10100-F	GACGAGCAACAACAACAA
Cluster-2384.10100-R	CCAACAGCAGAAGAAGAAG
Cluster-2384.12950-F	TCAACAGTCAGCATAGAGAA
Cluster-2384.12950-R	CCATTGCGATATTCCAACA
Cluster-2384.8096-F	CCTTGTTGGTAACGGATTC
Cluster-2384.8096-R	ACGGATTGGACATTGATTG
Cluster-2384.8883-F	TCATCATCTCAGTCCTTATCC
Cluster-2384.8883-R	AGTTGAAGCGACGAGTAA

Table 1. Continued

Primer name	Primer sequence (5′–3′)
ACT-F	CCACACTGTCCCCATTTATGAA
ACT-R	CGCGACCAGCCAAATCC

bodies of *D. galeata* were respectively collected as 1 sample in the control (BH, BT, and BR) and fish kairomone (PH, PT, and PR) groups. Each sample had 2 replicates. All samples were immediately frozen in liquid nitrogen and stored at –80 °C.

RNA extraction and cDNA library construction

Total RNA was extracted from 6 samples consisting of 6 different parts of *D. galeata* bodies (BH, BT, BR, PH, PT, and PR) under 2 concentrations of FK using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA library construction and Illumina sequencing of the 6 samples were performed at Novogene Bioinformatics Technology Co., Ltd., Beijing, China. Additionally, mRNA was purified from total RNA using oligo (dT) magnetic beads. First-strand cDNA was generated using random hexamer-primed reverse transcription and second-strand cDNA was synthesized using RNase and DNA polymerase I. After the end repair and ligation of adaptors, the library fragments were purified using the AMPure XP system (Beckman Coulter, Brea, CA, USA).The products were amplified by PCR to create a cDNA library, and library quality was assessed using a Bioanalyzer 2100 system (Agilent, Santa Clara, CA, USA) (Zhang et al. 2016).

Clustering and sequencing

Clustering of index-coded samples was performed with a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, USA). After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform (Illumina) and paired-end reads were generated.

De novo assembly of short reads and gene annotation

Clean data (clean reads) for the BH, BT, BR, PH, PT, and PR samples were obtained by removing low quality reads and those containing adapters andpoly-N sequences from the raw data. Transcriptome de novo assembly was conducted with these short reads using the assembling program Trinity (Grabherr et al. 2011) with min_kmer_cov set to 2 by default and all other parameters kept at their default settings. The resulting sequences were named unigenes. The unigenes above 150 bp were annotated by BLASTX searching in NCBI non-redundant (Nr), Swiss-Prot, KEGG, and COG databases, with an *E*-value threshold of 10^{-5}. Functional annotation by Gene Ontology (GO) was performed using the Blast2GO program (http://www.blast2go.org/) (Conesa et al. 2005), and GO functional classification of unigenes was performed using WEGO software (BGI, Shenzhen, China). Similarity searches for unigenes were performed using the NCBI-BLAST network server (http://blast.ncbi.nlm.nih.gov/).

Differentially expressed genes and GO enrichment analysis

Differentially expressed genes (DEGs) between 2 different parts of the body (PT vs. BT, PH vs. PR, PH vs. BH, BH vs. BR, PH vs. PT, and BH vs. BT) of *D. galeata* was analyzed

Table 2 Name and sequence of primers used in the experiments

Primer name	Forward (5′–3′)	Reverse (5′–3′)
Dagcut	CCCAAGCTTATGACTCATGCACGCAGT	CCGCTCGAGTTATTCATACAAATTATTATAGCGTCT
EGFP	CGCGGATCCATGGTGAGCAAGGGCGAGG	CCGCTCGAGTTACTTGTACAGCTCGTCCATGCCG

Dagcut and *EGFP* are interference primer.

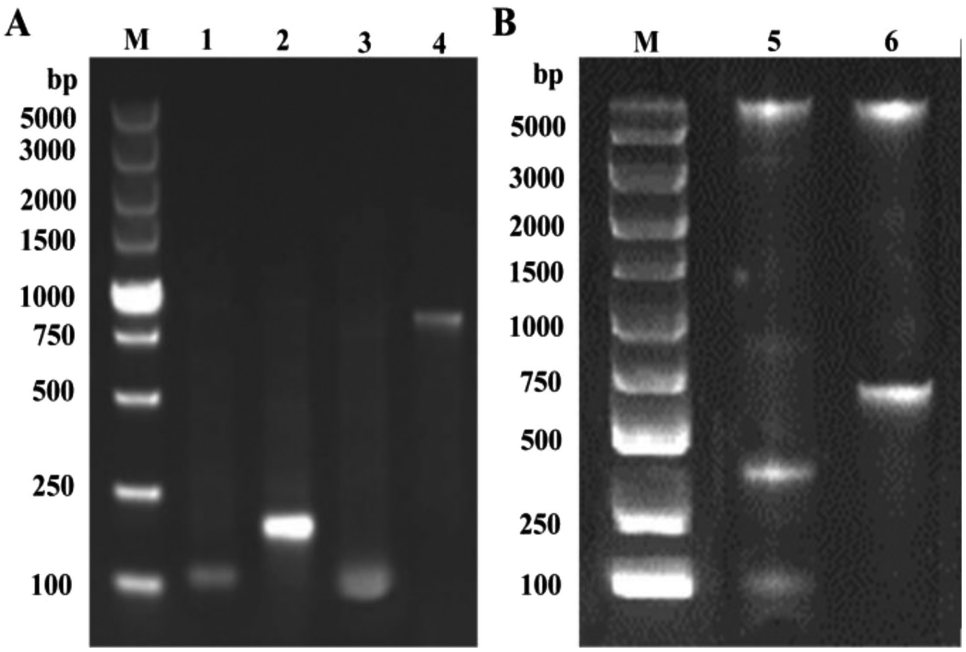

Figure 2 Induced expression of L4440-EGFP and L4440-Dagcut fragments. (A) Induced expression levels of HT115 strain carrying L4440 vector plasmid and L4440-EGFP recombinant plasmid (cited from Qi et al. 2023); (B) Induced expression levels of HT115 strain carrying l4440-Dagcut recombinant plasmid. M: DNA molecular weight standard; 1: HT115 strain carrying L4440 vector plasmid was not induced; 2: induced product of HT115 strain carrying L4440 vector plasmid; 3: HT115 strain carrying l4440-EGFP recombinant plasmid was not induced; 4: induced product of HT115 strain carrying l4440-EGFP recombinant plasmid; 5: HT115 strain carrying l4440-Dagcut recombinant plasmid was not induced; 6: Induced product of HT115 strain carrying l4440-Dagcut recombinant plasmid.

using the DEGseq 2 package (Storey 2003). Each sample had 2 replicates. BH, BT, and BR stand for the helmet, tail spine, and body in the control group, respectively, whereas PH, PT, and PR stand for the helmet, tail spine, and body in the fish kairomone group, respectively. *P*-values were adjusted using *Q*-values. *Q*-value < 0.05 and |log2(fold change)| > 1 were set as the thresholds for significantly differential expression (Zhang et al. 2016). GO enrichment analysis of DEGs was performed using the GOseq package based on the Wallenius non-central hyper-geometric distribution (Young et al. 2010).

In previous experiments, the helmets and tail spines of *D. galeata* were significantly affected under the stress of FK. Moreover, some investigations had showed that different genes had specific expression sites in *Daphnia* (Liu et al. 2014; Kong et al. 2016; Qi et al. 2023) by whole in situ hybridization experiment. Therefore, in order to find the key genes to regulate the helmet and tail spine of *D. galeata*, DEGs were sought by the comparisons of transcriptome sequencing of different body parts.

Validation of DEGs using real time-PCR

Total RNA was extracted using the TRIzol reagent (TaKaRa, Dalian, China). An ultramicro-spectrophotometer (MD2000D, Biofuture, UK) was used to assess sample purity and RNA concentration. RNA was reverse-transcribed using the PrimeScript™ RT reagent kit (TaKaRa).

RT-qPCR was performed with 123 genes, which were selected from top 30 up-regulated DEGs in PT versus BT, PH versus PR, PH versus BH, BH versus BR, PH versus PT, and BH versus BT. qPCR was performed using a LightCycler® 96 (Roche Diagnostics Gmbh, Basel, Switzerland) with a mixture of 5.0 μL AceQ qPCR SYBR Green Master Mix (Vazyme,Nanjing,China), 0.2 μL of each primer, 1.0 μL of sample cDNA, and 3.6 μL of RNase-free dH_2O. Amplification was performed using a degeneration step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A melting curve was utilized to detect a single primer-specific peak at 93 °C for 30 s and 60 °C for 45 s. The qPCR primers were designed using Beacon Designer 7.9 (Premier Biosoft International, Palo Alto, CA, USA) and are listed in Table 1. DsimACT (actin) was used as the reference gene. All reactions were performed in triplicate. The relative quantification results were analyzed using the Ct method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen 2001).

dsRNA preparation

One cuticle protein gene (Cluster-2384.10979, *Dagcut*) may be closely related to the morphological defense of

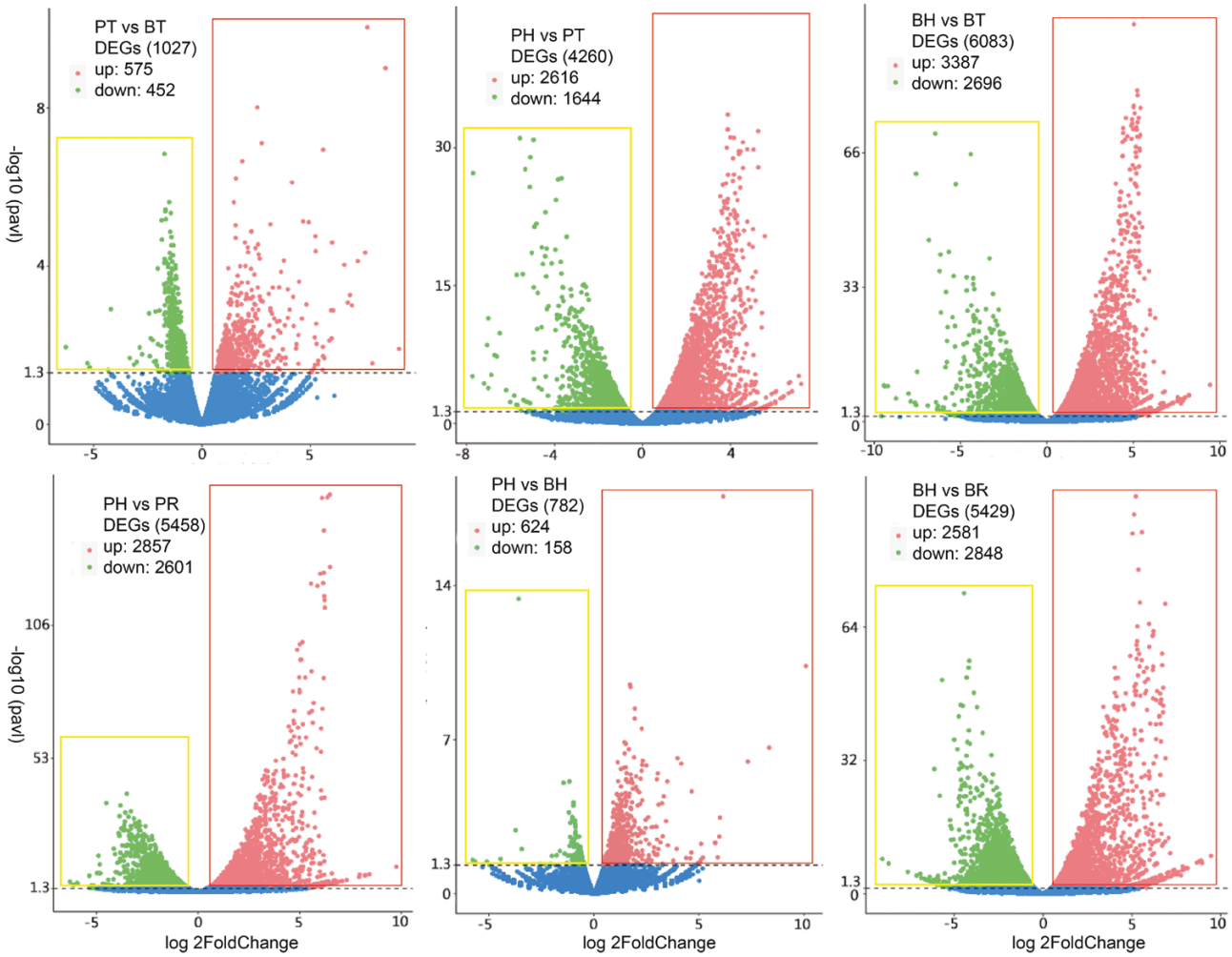

Figure 3 Volcano plot of DEGs of *Daphnia galeata* in PT versus BT, PH versus PR, PH versus BH, BH versus BR, PH versus PT, and BH versus BT. Based on *Q*-value < 0.05 and |log2(fold change)| > 1 as the thresholds for significantly differential expression. BH: the helmet for control group, PH: the helmet for FK group; BT: the tail spine for control group, PT: the tail spine for FK group; BR: the body for control group, PR: the body for FK group. Below the dashed line (blue): represents no DEGs; Above the dashed line: red box represents up-regulated DEGs, and yellow box represents down-regulated DEGs. The *Dagcut* gene was located in up-regulated region in PT versus BT group (the long2Foldchange: 3.5).

D. galeata against FK. Primers were designed according to the transcriptome data and *EGFP* (Enhanced Green Fluorescent Protein) plasmid sequences (Table 2). The PCR amplification conditions were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 55–60 °C for 15 s, and 72 °C for 40 s, and then lastly, 72 °C for 5 min. PCR products were detected using 1% agarose gel electrophoresis. Subsequently, *Dagcut*/*EGFP* genes from the PCR products were sub-cloned into the pEASY-Blunt3 cloning vector (TransGen, Beijing, China), and sequenced at General Biology, Nanjing, China.

For the next step, the cloned *Dagcut*/*EGFP* genes had to be inserted into a vector before transformation into *E. coli*, and the L444 vector was chosen. The L4440 vector contains 2 T7 promoters that can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) to produce dsRNA of the sequence ligated between these promoters. The expression vectors L4440 and the pEASY-Blunt3-Dagcut/EGFP plasmid were digested using restriction enzymes *Hind* III and *Xho* I (TaKaRa), and then ligated. The L4440 constructs were transformed into *E. coli* DH5α cells (Sangon Biotech, Shanghai, China) and the vector was confirmed by sequencing (General Biology). After sequencing, the L4440-Dagcut plasmid was transformed into *E. coli* HT115 cells—a strain deficient in RNase III and capable of efficient production of dsRNAs. The transformed cells were cultured overnight in LB medium containing 100 μg mL^{-1} ampicillin (Sangon Biotech) and 12.5 μg mL^{-1} tetracycline (Sangon Biotech) for the *Dagcut* RNAi experiments. Isopropyl IPTG (1.0 mM, Sangon Biotech) was added to induce T7 RNA polymerase and the subsequent production of dsRNA of the target sequence. The expression fragments of the L4440 vector plasmid, L4440-EGFP recombinant plasmid, and L4440-Dagcut recombinant plasmid induced by IPTG were 163, 913, and 459 bp, respectively. In agarose gel electrophoresis (1%), the L4440 vector plasmid and L4440-EGFP recombinant plasmid were approximately 150 and 900 bp in size (Figure 2A), and the L4440-Dagcut recombinant plasmid was approximately 500 bp in size (Figure 2B). This suggests that the induced dsRNA was successful. Positive clones were selected and cultured in liquid LB medium containing ampicillin and tetracycline, until the value of OD_{600} was between 0.6 and 0.8. dsRNA was stored in 25% glycerol and frozen at −80 °C.

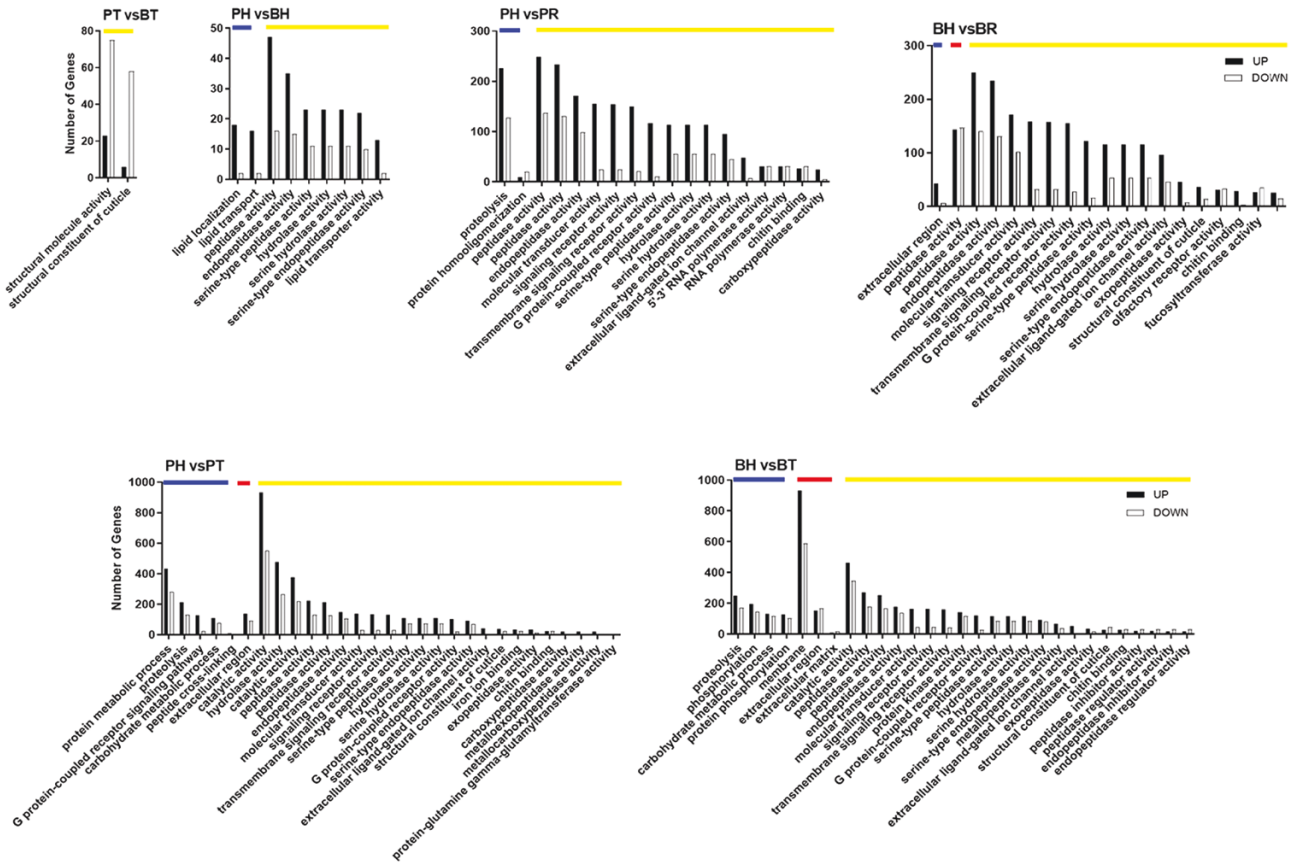

Figure 4 GO enrichment analysis of DEGs in PT versus BT, PH versus PR, PH versus BH, BH versus BR, PH versus PT, and BH versus BT. Blue: BP represents biological process. Red: CC represents cellular component. Yellow: MF represents molecular function. BH: the helmet for control group, PH: the helmet for FK group; BT: the tail spine for control group, PT: the tail spine for FK group; BR: the body for control group, PR: the body for FK group.

RNA interference

Two different food groups were used in this study: *D. galeata* in the Dagcut group was fed 2% dsRNA-Dagcut + 98% *T. obliquus* as a treatment group, and *D. gelaeat* in the EGFP group was fed 2% dsRNA-EGFP+ 98% *T. obliquus* as a control group. The total food biomass was provided with 20 mg L^{-1} wet weight for each treatment. There were 90 replicates for each food treatment and each replicate contained 30 *D. galeata* newborns. Each *D. galeata* newborn was cultured in a 50 mL beaker containing 40 mL of culture medium. The culture medium is filtered tap water that has been aerated for more than 48 h. The experiments were conducted in an illumination incubator at 25 ± 1 °C with a 12 h: 12 h light/dark cycle and a light intensity of 2,500 lx. The culture medium was replaced every day. Newborns (birth time < 24 h) of *D. galeata* in the third generation were collected for *E. coli* feeding experiments. The RNAi experiment lasted for 9 days. During the experiment, helmet, body, and tail spine lengths at each instar were measured under a microscope, and developmental abnormalities in the late embryos and neonates were also observed. At the end of the experiment, all *D. galeata* were immediately frozen in liquid nitrogen and stored at −80 °C. During the experimental period, neonates produced by females were promptly removed from the beakers.

After RNAi, the expression levels of related genes were determined by qPCR and were then calculated using the Ct method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen 2001).

Whole *in situ* hybridization

To prepare probes for in situ hybridization according to the ORF of the *Dagcut* gene, the sequences of specific primers were designed as follows: ISH-Dagcut-Forward: CCCCTCGAGGTGTCTACCTTACTTATGGCTC; ISH-Dagcut-Reverse: CCGAAGCTTATGACTCGATATGGAGG, with a length of 150 bp. The target fragment was synthesized according to primer sequences. After sequencing, the positive bacterial clones were amplified and cultured, and then the Blunt3-ISH-Dagcut vector plasmid was extracted. The concentration and purity of the plasmids were determined using a NanoDrop spectrophotometer (MD2000D, Biofuture, USA). The linearized plasmid was obtained by restriction digestion with *Hind* III or *Xho* I. The digested DNA fragments were purified and used as templates for sense and antisense probes, respectively. RNA probes were synthesized using the DIG RNA Labeling Kit (SP6/SP7; Roche), and the probe cDNA template was digested using DNase (RNase-free). In addition, 1/9 volume of 5 M LiCl and 2 volumes of absolute ethanol were added and incubated overnight at −20 °C. Afterwards, RNA pellets were washed twice with 75% ethanol, and residual ethanol was removed by drying. Finally, the RNA pellets were resuspended in 30 µL diethylpyrocarbonate water, and 1 µL of RNA inhibitor (20 U) was added. Aliquots of RNA solution (1 µL) were added and electrophoresed, and their concentrations were measured. The remaining RNA probes were stored at −20 °C.

Table 3 Twenty-four DEGs related to morphology in *Daphnia galeata*

Gene ID	Gene length	NR description	PFAM description
Cluster-2384.9683	2304	Hypothetical protein DAPPUDRAFT_262644	Trypsin
Cluster-2384.3055	1911	Hypothetical protein DAPPUDRAFT_328578	–
Cluster-2384.9311	5111	Hypothetical protein DAPPUDRAFT_318106	Trypsin
Cluster-2384.2380	2100	Hypothetical protein DAPPUDRAFT_302529	–
Cluster-2384.4388	1093	Hypothetical protein DAPPUDRAFT_112082	–
Cluster-2384.5440	894	Hypothetical protein DAPPUDRAFT_327500	–
Cluster-2384.10979	2139	Hypothetical protein DAPPUDRAFT_327708	Cuticle protein
Cluster-2384.16890	718	Hypothetical protein DAPPUDRAFT_108524	–
Cluster-2384.10346	1365	Glutamine gamma-glutamyltransferase Z	Transglutaminase family
Cluster-2384.4417	1688	Hypothetical protein DAPPUDRAFT_230618	Papain family cysteine protease
Cluster-2384.8245	366	Hypothetical protein DAPPUDRAFT_346977	–
Cluster-2384.13779	5023	Hypothetical protein DAPPUDRAFT_300683	Leucine Rich repeat
Cluster-2384.11140	1384	Hypothetical protein DAPPUDRAFT_117948	–
Cluster-2384.12328	7935	Hypothetical protein DAPPUDRAFT_54362	Ankyrin repeat
Cluster-2384.12999	1209	Hypothetical protein DAPPUDRAFT_309279	–
Cluster-2384.8263	1575	Ferritin 1-like protein A	Ferritin-like domain
Cluster-2384.3878	1223	C1qdc1-like protein	–
Cluster-2384.6917	2101	Hypothetical protein DAPPUDRAFT_231459	Trypsin
Cluster-2384.7946	1246	Hypothetical protein DAPPUDRAFT_192539	Papain family cysteine protease
Cluster-2384.9831	925	Ferritin	Ferritin-like domain
Cluster-2384.7175	951	Hypothetical protein DAPPUDRAFT_308767	–
Cluster-2384.16699	2361	Hypothetical protein DAPPUDRAFT_194683	Galactosyltransferase
Cluster-2384.10100	3102	Hypothetical protein DAPPUDRAFT_101743	–
Cluster-2384.12950	1235	Hypothetical protein DAPPUDRAFT_94570	Domain of unknown function (DUF1984)
Cluster-2384.8096	5328	Acidic mammalian chitinase	Chitin binding Peritrophin-A domain
Cluster-2384.8883	2850	Putative transcriptional factor distal-less protein	Homeodomain

According to the RNAi feeding protocol, 50 *D. galeata* from the EGFP and Dagcut groups were collected. All samples were fixed in 4% paraformaldehyde (4% PFA) overnight, and anhydrous methanol was used to replace 4% paraformaldehyde at –20 °C. Whole-mount in situ hybridization was carried out according to previously published methods (Liu et al. 2014; Kong et al. 2016) with some modifications. Specimens stored at –20 °C were rehydrated gradually with methanol-PBST and digested with 10 μgmL^{-1} proteinase K (Solarbio, Beijing, China) at 37 °C for 12 min. After pre-hybridization at 68 °C for 2.5 h, 100 μL of RNA probe (diluted 1: 100) was added and incubated overnight at 70 °C. The specimens were blocked in MAB block solution for approximately 2 h at room temperature with slow shaking and incubated again at 4 °C for 13 h after an anti-DIG antibody (diluted 1: 5,000; Roche) was added. Subsequently, the antibody solution was washed with MABT buffer. At room temperature, NBT liquid dye (Roche) was used to dye the specimens for 15–30 min, and then the specimens were fixed with 4% PFA for 20 min. Hybridization was observed and recorded under a microscope.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software. Significant differences in relative *Dagcut* gene mRNA expression levels between 2 body parts (PT vs. BT, PH vs. PR, PH vs. BH, BH vs. BR, PH vs. PT, and BH vs. BT) were analyzed using Tukey's multiple comparison test (HSD). All data are shown as mean ± SEM in this study.

Results

DEGs and GO annotation

Among the DEGs in *D. galeata*, the number of up-regulated and down-regulated genes were 575 and 452 in PT versus BT, 2,857 and 2,601 in PH versus PR, 624 and 158 in PH versus BH, 2,581 and 2,848 in BH versus PR, 2,616 and 1,644 in PH versus PT, and 3,387 and 2,696 in BH versus BT, respectively (Figure 3).

To investigate the functions of these DEGs, we analyzed 1027 DEGs in PT versus BT, 5,458 in PH versus PR, 782 in PH versus BH, 5,429 in BH versus BR, 4,260 in PH versus PT, and 6,083 in BH versus BT using the GO enrichment system (Q-value < 0.05). In the PT versus BT comparison, up-regulated and down-regulated genes were mainly concentrated in the structural construct of the cuticle (6 and 58, respectively) and structural molecular activity (23 and 75, respectively). In the PH versus PR comparison, both up-regulated and down-regulated genes were mainly concentrated in peptidase activity (248 and 137, respectively) and peptidase activity acting on L-amino acid peptides (233 and 131, respectively). In the PH versus BH comparison, up-regulated and down-regulated genes were mainly concentrated in peptidase activity acting on L-amino acid peptides (47 and 16, respectively) and endopeptidase activity (35 and 15, respectively). In the BH versus BR comparison, up-regulated

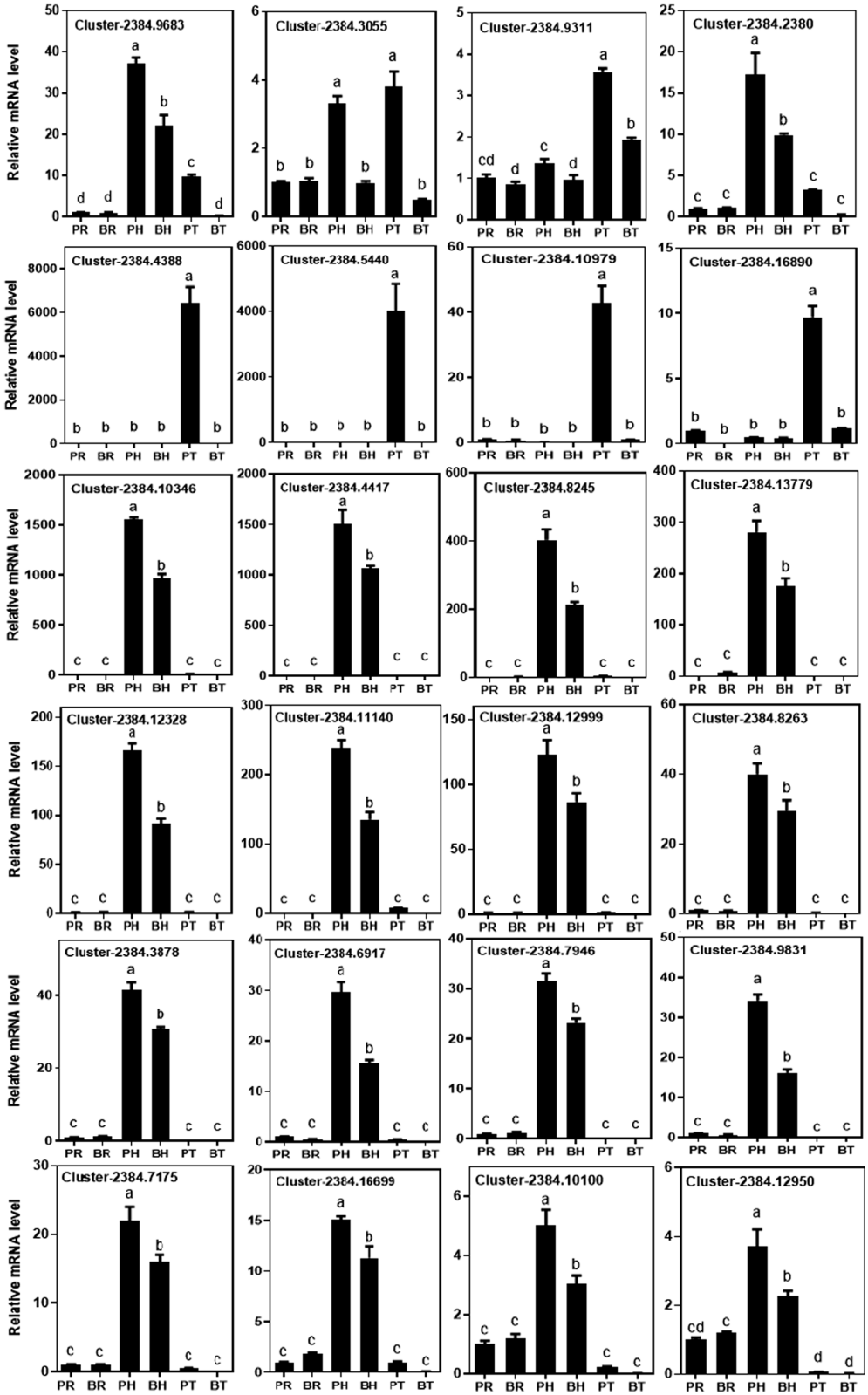

Figure 5 qPCR results of DEGs related to tail spines. Significant differences between the PR, BR, PH, BH, PT and BT groups were indicated by small letters ($P < 0.05$). BH: the helmet for control group, PH: the helmet for FK group; BT: the tail spine for control group, PT: the tail spine for FK group; BR: the body for control group, PR: the body for FK group.

genes were mainly concentrated in peptidase activity acting on L-amino acid peptides (235) and endopeptidase activity (249), and the down-regulated genes were mainly concentrated in the extracellular region (147) and peptidase activity (140). In the PH versus PT comparison, up-regulated and down-regulated genes were mainly concentrated in protein metabolic processes (434

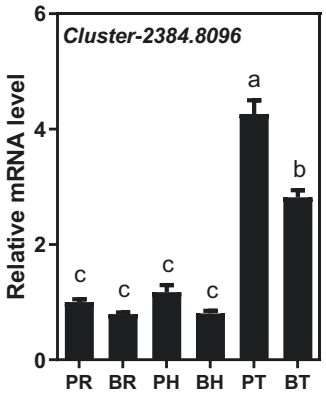

Figure 6 qPCR results of some published genes related to morphology genes. Cluster-2384.8096 (*Dagcut* gene) in *Daphnia galeata* (Chitin-related enzymes (Christjani et al. 2016)) may be related to morphology in other *Daphnia* species.

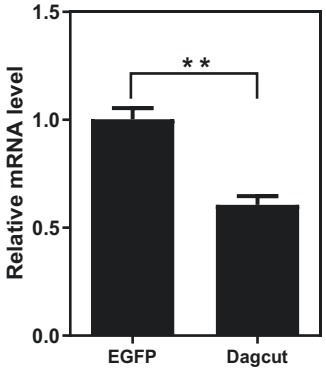

Figure 7 Expression of the *Dagcut* gene in *Daphnia galeata* after RNAi. Significant difference between the EGFP and Dagcut group was indicated (**$P < 0.01$).

and 282, respectively) and catalytic activity acting on a protein (376 and 222, respectively). In the BH versus BT comparison, up-regulated and down-regulated genes (933 and 589, respectively) were mainly concentrated in the membrane (Figure 4).

Validation of DEGs

To screen candidate genes related to the morphology of *D. galeata*, a total of 123 genes were obtained from the top 30 up-regulated DEGs among all tissue/condition comparisons. Through qPCR analysis, 24 candidate genes related to the morphological defense of *D. galeata* were confirmed (Table 3). The relative expression levels of 4 genes (Cluster-2384.9683, Cluster-2384.3055, Cluster-2384.9311, and Cluster-2384.2380) in PH were significantly higher than that in BH, PR, and BR ($P < 0.05$), and these 4 genes in PT were significantly higher than those in BT, PR, and BR ($P < 0.05$), suggesting that these genes may be involved in the morphological changes (including the helmet and tail spine) of *D. galeata* (Figure 5). Two known trypsin genes (Cluster-2384.9683 and Cluster-2384.9311) were identified.

The expression levels of 4 genes (Cluster-2384.4388, Cluster-2384.5440, Cluster-2384.10979, and Cluster-2384.16890) in PT were significantly higher than those in BH, BT, BR, PH, and PR ($P < 0.05$), and these genes may be involved in the tail spine changes in *D. galeata* (Figure 5). Among these genes, 1 known cuticle protein gene (Cluster-2384.10979) was observed. The expression levels of the other 16 genes in PH were significantly higher than those in BH, BT, BR, PT, and PR ($P < 0.05$) (Figure 5); among these, 2 ferritin genes (Cluster-2384.8263 and Cluster-2384.9831) and 1 C1qDC protein gene (Cluster-2384.3878) may be involved in helmet changes in *D. galeata*, which are associated with immunity and defense.

In addition, 2 known genes (Cluster-2384.8096: acidic mammalian chitinase; Cluster-2384.8883: putative transcriptional factor distal-less protein) that may be involved in morphological changes were also found in the *D. galeata* transcriptome (Table 3). The qPCR results showed that the expression levels of Cluster-2384.8096 and Cluster-2384.8883 in PH and PT were higher than those in BH and BT, respectively ($P < 0.05$; Figure 6), suggesting that the 2 genes were involved in the morphological defense changes of both helmet and tail spines of *D. galeata* with the presence of FK.

Dagcut gene expression and morphology changes of *D. galeata* after RNA interference

Compared to the EGFP group, the relative expression levels of the *Dagcut* gene of *D. galeata* in the Dagcut group decreased by 33.46% (Figure 7). Multiple comparisons showed that the relative expression levels of the *Dagcut* gene in the Dagcut group were significantly lower than those in the EGFP group (Figure 7).

During the RNAi experiment, in the Dagcut group, 37.5% of the 5 to 7 instar individuals of *D. galeata* died because of an inability to molt normally (Figure 8F). In the Dagcut group, bent tail spines were detected in both the living and dead offspring of *D. galeata* during the second reproduction cycle (Figure 8E). Moreover, the tail spines of a few mothers also showed a curved structure (Figure 8C,D). However, in the EGFP group (control), the tail spine morphology of all mothers and their offspring of *D. galeata* were normal (Figure 8A,B). After RNAi, whole-mount in situ hybridization showed that the *Dagcut* gene in parthenogenetic females was mainly expressed on the tail spines of *D. galeata* (Figure 9).

Discussion

Under predation pressure, cladocera can initiate inducible defenses and produce morphological changes (Tollrian 1990; Rabus et al. 2012, 2013; An et al. 2018). Cuticle, which covers the entire outer surface in crustaceans, provides the first defence against physical or chemical damages (including predator kairomones). Cuticle proteins and chitin filaments are the primary components of the cuticle (Yang et al. 2020). In the presence of the predator *T. cancriformis*, a bulky morphotype (longer body length and width, and an elongated tail spine) of *D. magna* has been observed (Rabus et al. 2012, 2013). In the presence of the predator kairomones, some investigations have shown that cuticle-related genes are involved in the formation of neckteeth in *D. pulex* (Rozenberg et al. 2015; An et al. 2018). In addition, carapace morphologic defense and cuticle hardening of *D. magna* were also observed after exposure to the predator kairomone (Otte et al. 2014). Ultrastructural observations have shown that the cuticle of *D. magna* exposed to *T. cancriformis* was observed to be harder and thicker than that of control daphnids (Rabus et al. 2013). However, there are no studies looking at the key functional genes related to

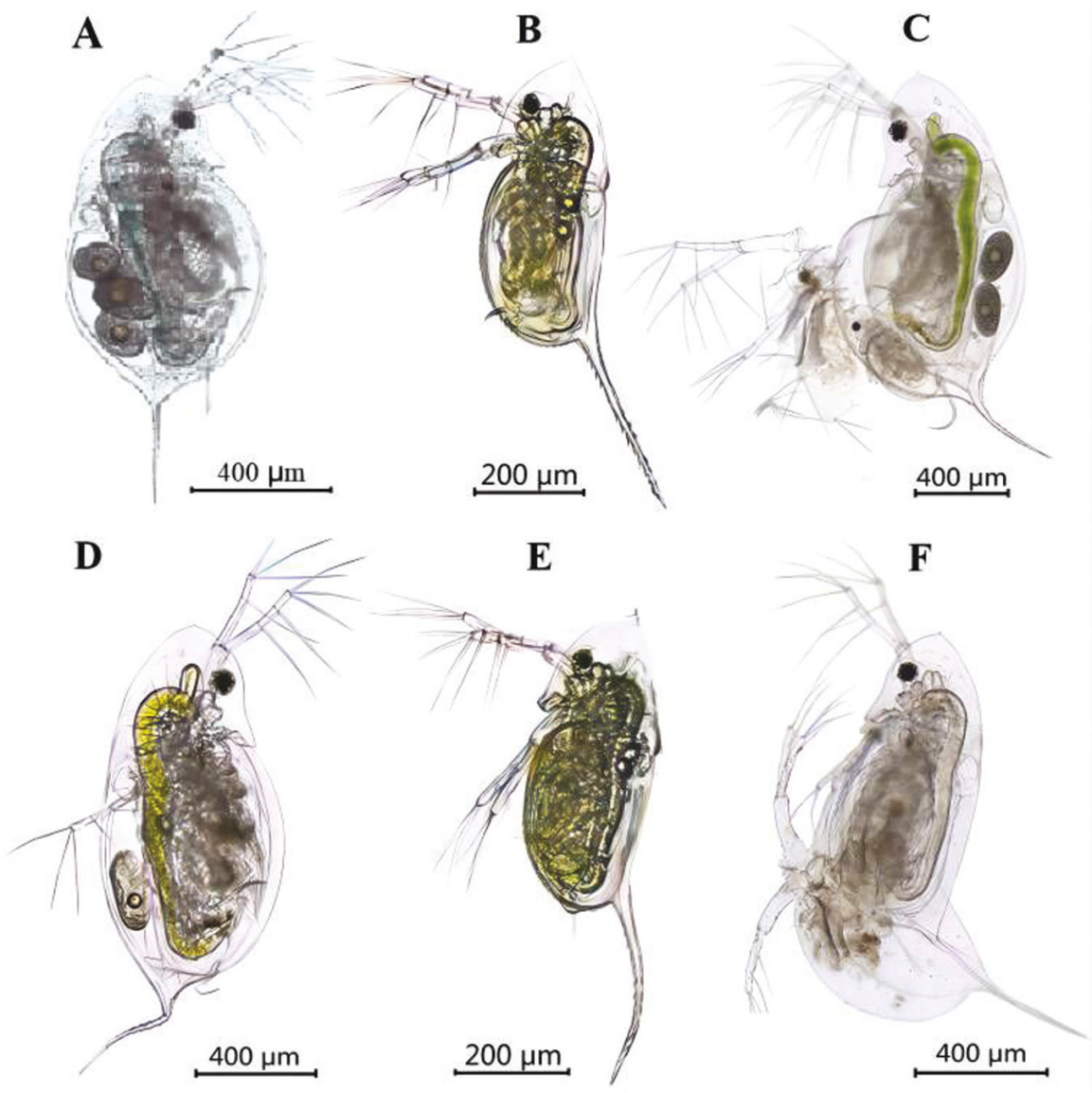

Figure 8 Morphological observation of *D. galeata* before and after RNA interference. (A) Normal pregnant individual; (B) Normal offspring; (C) The dead individual at first brood because of the molting failure, which produced dead juveniles with a curved tail spines in the Dagcut group; (D) The individual at maturity in the Dagcut group; (E) The offspring at first reproduction in the Dagcut group; (F) a dead larva because of failure to molting in the Dagcut group.

other morphological changes (e.g., helmet and tail spine) in *Daphnia* species under the fish-predator kairomone. In this study, 24 candidate genes related to the morphology (e.g., helmet and tail spine) of *D. galeata*were screened in the presence of *A. mobilis* kairomones. Determining the functions of these candidate genes will further reveal the molecular mechanisms of predator-induced defenses.

Generally, changes in the cuticle carapace structure can provide effective protection against *Daphnia*. Morphological changes in the cuticle may be related to predator-induced defenses. Rabus et al. (2013) found significant relationships between the morphologies (such as body length, tail spine length, and relative tail spine length) of *D. magna* and the predator kairomones of *T. cancriformis*. In the presence of *T. cancriformis*, some proteins (e.g., muscle proteins, cuticle proteins, and chitin-modifying enzymes) may be directly involved in carapace stability of *D. magna* (Otte et al. 2014), and then cause inducible defenses by morphological change of carapace and cuticle hardening. When predator *Chaoborus* larvae are present, some effector genes (including cuticle genes) are mostly associated with phenotypic changes including morphological alterations (e.g., the formation of protective structures and cuticle strengthening) in *D. pulex* (Rozenberg et al. 2015). Small-molecule neurotransmitter pathways are potentially involved in the development of inducible defenses (An et al. 2018). Neurohumoral transmission may regulate necktooth formation and cuticle strengthening in *D. pulex* under the activity of predator kairomones (Weiss et al. 2012; Rozenberg et al. 2015). The formation of neckteeth in *D. pulex* is found to be related to genes in endocrine and juvenile hormone pathways (Miyakawa et al. 2010; Weiss et al. 2012; Miyakawa et al. 2013). In this study, in PT versus BT, the GO enrichment results showed that the up-regulated genes were mainly involved in the function of

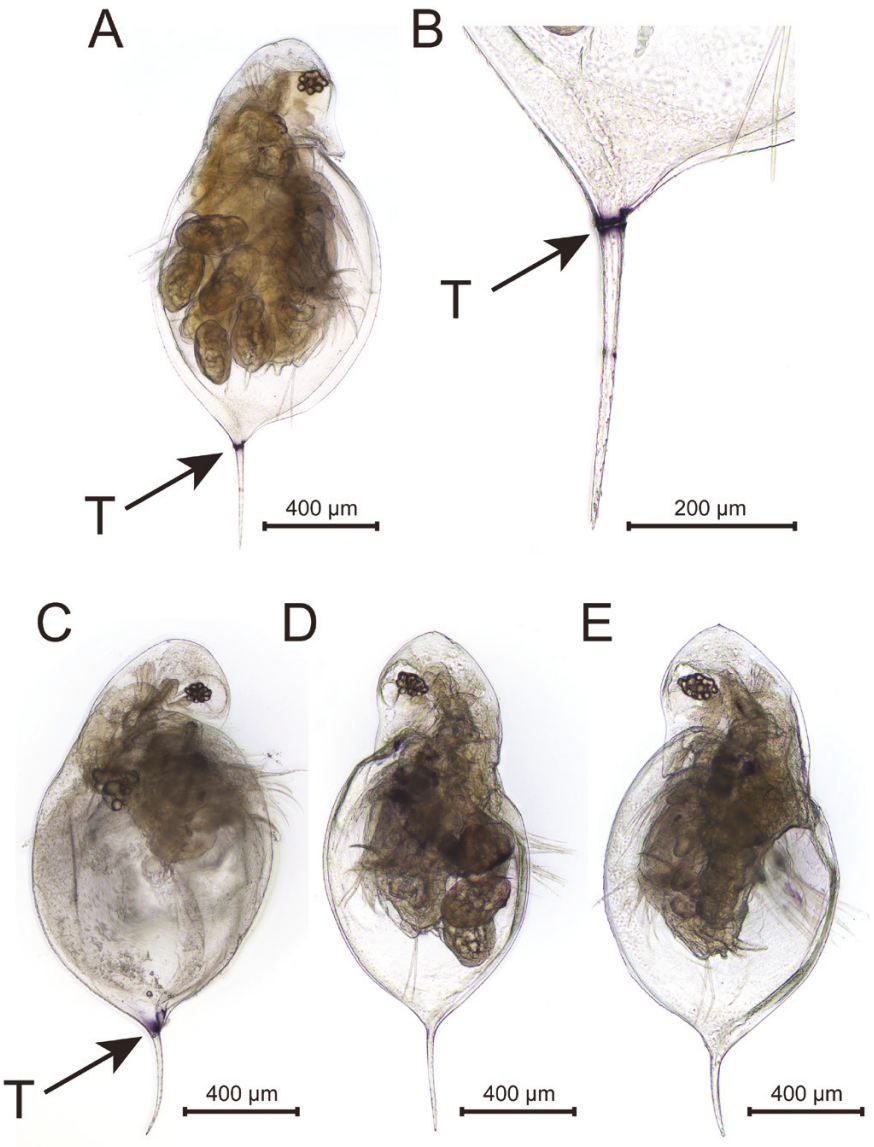

Figure 9 Expression of *Dagcut* mRNA in *Daphnia galeata* was determined by whole-mount in situ hybridization after RNAi. (A and C) Antisense probe of the *Dagcut* gene before RNAi; (B) the magnification of A in part; (D) Antisense probe of the *Dagcut* gene after RNAi; (E) Sense probe. Violet area shows positive signals. T: Tail spine.

cuticle structural components in *D. galeata*. The expression level of Cluster-2384.10979 (*Dagcut* gene) in PT was significantly higher than that in the other 5 parts (PH, BH, PR, BR, and BT), and Cluster-2384.10979 was annotated cuticle protein (Table 3). This suggests that FK may induce cuticle genes expression in the tail spine of *D. galeata* to adapt to environmental changes. Christjani et al. (2016) found that chitin-related genes are also involved in the development of morphological defenses in *Daphnia*. The exoskeleton of cladocerans consists not only of cuticle proteins but also chitin structures (Schlotz et al. 2012). In the process of the molting of insects, chitinase is an important factor for degrading chitin and breaking down old cuticle (Merzendorfer and Zimoch, 2003). Moreover, in this study, the expressions of 2 morphology-related genes (Cluster-2384.8096 and Cluster-2384.8883) in PT were significantly higher than that in BT, which were related to inducible defenses in *D. pulex* (Hiruta et al. 2013; Christjani et al. 2016). Therefore, further investigations on the functions of chitinase genes are necessary to understand the formation of defense morphisms in cladocerans.

RNA interference (RNAi) has been extensively used to silence target genes in cladocerans (Chen et al. 2014; Schumpert et al. 2015; Street et al. 2019; Qi et al. 2023). The function of the molting gene *CYP302A1* in *D. sinensis* has also been analyzed using RNAi (Qi et al. 2023). In this study, the *E.coli* HTll5 strain containing dsRNA was used to feed *D. galeata* to explore the changes in gene expression of *Dagcut* and phenotype, and to analyze the function of the *Dagcut* gene. Our results showed that the *Dagcut* gene expression levels of *D. galeata* in the Dagcut group were significantly down-regulated compared to the EGFP group. In the Dagcut group experiment, the tail spines of the mother at first brood or the offspring produced during the second reproductive cycle in *D. galeata* were curved, and 37.5% of the 5 to 7 instars of *D. galeata* died because of a failure molting. These results suggest that the *Dagcut* gene may be directly involved in the morphological development (tail spine) of *D. galeata*. Kim et

al. (2011) found that exposure to fenoxycarb (a model juvenile hormone) produced significant changes in the expression level of *Cuticle 12* in *D. magna*, and developmental abnormalities such as poorly developed second antennae and curved tail spines were also observed. These results suggest that cuticle-related genes play an important role in maintaining the tail spine phenotype in *Daphnia*. Other studies have shown that cuticle protein genes are closely related to insect molting and metamorphosis development (Mun et al. 2015; Wu et al. 2016). The gene encoding the cuticle protein is associated with the molting inhibition hormone in *D. magna* (Soetaert et al. 2007). In addition, in this study, the whole in situ hybridization also showed that *Dagcut* was expressed mainly in the tail spine of *D. galeata*. Therefore, the *Dagcut* gene may play key roles in the development of tail spine morphology and the molting of *D. galeata*. It is likely that the cuticle-related genes may be regulated by a transcription factor activated by neurotransmitters or the hormonal system. Further studies on the molecular mechanisms triggering the activation of morphology-related genes in *Daphnia* are necessary in the presence of predator kairomones.

In conclusion, FK could induce morphological defenses in *D. galeata*. In this study, the helmet, tail spine, and body of *D. galeata* were cut to screen DEGs by transcriptome sequences induced by FK for the first time. A total of 123 genes were obtained from the top 30 up-regulated DEGs among all 6 tissue/condition comparisons. Through qPCR analysis, 24 candidate genes related to the morphology (e.g., helmet and tail spine) of *D. galeata* were screened, among which the function of a candidate gene (Cluster-2384.10979, *Dagcut*) related to the formation of tail spine was analyzed using RNAi technology. After RNAi, compared with the EGFP group, the expression level of the *Dagcut* gene in the Dagcut group showed a significant decrease. Moreover, during the experiment, the tail spines of some late embryos and offspring of *D. galeata* were found to be curved in the Dagcut group. In whole-mount in situ hybridization experiment, an obvious signal site was detected on the base of the tail spine of *D. galeata* before RNAi, whereas the expression signal disappeared after RNAi. Our results suggest that the *Dagcut* gene may play an important role in tail spine formation in *D. galeata*, and provide a theoretical basis for studying the molecular mechanism of the morphological plasticity in cladocera in the future.

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Conflict of interest

We declare that we have no conflicts of interest.

Author contributions

Daogui Deng conceived the study. Yaqin Cao, Yajie Zhao, Huiying Qi, Jinfei Huang and Fucheng Zhu collected the samples and the data. Yaqin Cao and Wenping Wang performed the analyses, Yaqin Cao wrote the first draft of the manuscript. Daogui Deng and Wenping Wang reviewed and revised the manuscript. All authors have approved and contributed to the final manuscript.

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