IDENTIFICATION OF ANTIOXIDANT GENES IN THE OSTRACOD Heterocypris incongruens THROUGH DE NOVO TRANSCRIPTOME SEQUENCING

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Despite the ecotoxicological value of the freshwater ostracod Heterocypris incongruens, there is little nucleotide sequence information on this species. With the aim to develop transcriptome resources of this species, we performed a de novo transcriptome analysis using a high-throughput sequencing technology and determined the sequences of antioxidant gene, such as catalase (cat), glutathione peroxidase (gpx), glutathione S-transferase C-terminal domain-containing protein (gstcd), and superoxide dismutase [Cu-Zn] (sod). To provide an application example of expression analyses using the obtained genes, we examined the expressions of the obtained genes by quantitative PCR (qPCR) in H. incongruens exposed to urban road dust, as a model of contaminated environmental sample. The expressions of all the target genes remained unchanged in response to road dust during six days of exposure. Our transcriptome data and sets of stress response genes of H. incongruens should be useful references to investigate the mechanism of sediment toxicity and to aid linkage between environmental contamination and biological responses.

Key Words : Heterocypris incongruens, ostracod, antioxidant genes, de novo transcriptome, toxicity, road dust

1. INTRODUCTION

Sediment accumulates a wide range of anthropogenic chemicals, such as polychlorinated biphenyls (PCBs)1), polycyclic aromatic hydrocarbons (PAHs)2), pesticides3), herbicides3), pharmaceuticals and personal care products (PPCPs)4), and metals 5,6). Sediment contamination can pose a potential threat to aquatic and benthic ecosystems. To assess sediment quality, laboratory sediment toxicity tests using benthic species are effective tools that can provide direct and quantifiable evidence of biological consequences of sediment contamination4).

Sediment toxicity tests have been standardized for several freshwater invertebrate species, such as midges Chironomus riparius and Chironomus tentans5)–6), an oligochaete Lumbricus variegatus7,8), and an amphipod Hyalella azteca4,5,8). However, culturing and maintaining of such test organisms often entail laborious works and financial burdens to the testing laboratories. To address this problem, a culture/maintenance-free test using the freshwater benthic ostracod Heterocypris incongruens has been developed9,10). This test starts with dormant cysts, eliminating the need for culturing and maintaining of organisms. Since H. incongruens is a small invertebrate species (hatched neonates have a length of 200 μm)9), the test is space-saving and does not re-
quire a large volume of sediment samples. In addition to its user-friendliness, the test with *H. incongruens* has a similar sensitivity to contaminated sediment and metals compared with the tests with *C. riparius* and *H. azteca*. Thanks to these advantages, the sediment toxicity test using *H. incongruens* was standardized by the International Organization for Standardization (ISO)\(^\text{(15)}\). The standardized test has been applied to toxicity assessments of various contaminated samples, such as freshwater sediment\(^\text{(2,3,15)}\), nanomaterials\(^\text{(16)}\), and road dust\(^\text{(17)-19)}\).

The toxic endpoints of the standardized test are limited to six-day mortality and growth inhibition. Whereas new reproductive endpoints in a chronic test have been proposed by recent studies\(^\text{(19,20)}\), responses at the below-individual level have never been investigated in *H. incongruens* in the context of ecotoxicology. Biological responses at the below-individual level (e.g., molecular and cellular levels) allow the mechanistic understanding of toxic effects and the causal link between environmental contamination and biological responses\(^\text{(21)}\). In particular, antioxidant genes in aquatic species, such as catalase (*cat*) and glutathione peroxidase (*gpx*), have been widely used in environmental monitoring and toxicology\(^\text{(22,23)}\).

The objective of this study was to determine the nucleotide sequences of antioxidant genes in *H. incongruens*, and to show an application example of expression analyses using antioxidant genes to an environmental contaminated sample. This study comprised the following three parts: First, we performed a de novo transcriptome analysis of the ostracods exposed to zinc, to identify the following antioxidant transcripts: *cat, gpx*, glutathione S-transferase C-terminal domain-containing protein (*gstcd*), and superoxide dismutase [Cu-Zn] (*sod*). Second, we conducted exposure tests of urban road dust, as an environmental contaminated sample, to determine the sub-chronic toxicity value. Urban road dust is known to be lethally toxic to benthic organisms\(^\text{(17,18,24,25)}\) including *H. incongruens*. Finally, we carried out quantitative PCR (qPCR) experiments in *H. incongruens* exposed to urban road dust to show an application example of the expression analyses of the identified genes. Despite its ecotoxicological value, the sequence information on *H. incongruens* was limited to cytochrome c oxidase subunit I of mitochondrial DNA and ribosomal RNAs\(^\text{(26)}\). Therefore, the transcriptome data obtained in this study will become useful references for future ecotoxicological studies using this species.

### 2. MATERIALS AND METHODS

#### (1) Source of organisms and road dust sample

*Heterocypris incongruens* was purchased as dormant cysts in Ostracodtoxkit F (MicroBioTests Inc., Belgium). The cysts were hatched in synthetic moderately hard water\(^\text{(27)}\) at 25 °C for 52 h under a 24-h light condition. The hatched ostracods were fed with Spirulina (supplied with Ostracodtoxkit F) 4 h prior to their use in exposure tests.

Urban road dust was collected in our previous study\(^\text{(25)}\) (referred to as RD3 in the previous paper) from the road surfaces of highways around Tokyo, Japan by sweeping vehicles in October 2015. The collected sample was dried in air overnight, sieved through 2-mm stainless mesh, freeze dried, and stored at 4 °C until use. The concentrations of metals, 12 PAHs, and nicotine are shown in Table 1.

### (2) Exposure experiments

#### a) Zinc exposure

To obtain transcriptome sequences associated with oxidative stress, transcriptome analysis was performed using the ostracods exposed to zinc, as a model oxidative chemical, at a nominal concentration of 75 µg Zn/L for six days. The zinc exposure concentration was more than two times higher than Japanese water quality criteria (30 µg Zn/L); however, was less than one-eighth of six-day LC50 value of zinc for *H. incongruens* (656 µg Zn/L\(^\text{(28)}\)).

Zinc exposure was conducted according to ISO 14371 methods\(^\text{(14)}\). The exposure was initiated by transferring 10 hatched ostracods to each well of a six-well plastic microplate (AGC Techno Glass) containing a mixture of 1 mL reference sediment supplied by MicroBiotests, 2 mL zinc stock solution, and 2 mL green algae suspension (1.5×10^7 cells/mL of *Scenedesmus acutus* Meyen, NIES-94). The zinc stock solution at a nominal concentration of 150 µg Zn/L was prepared by the addition of ZnSO\(_4\)·7H\(_2\)O (Purity: > 99.5%, Wako Pure Chemicals, Japan) into

| Concentration (mg/kg-dry) |
|--------------------------|
| Cr                       | 123          |
| Ni                       | 37.3         |
| Cu                       | 133          |
| Zn                       | 783          |
| Cd                       | 0.34         |
| Pb                       | 58.7         |
| Σ12 PAHs \( a \)         | < 0.37       |
| Nicotine \( b \)         | 0.76         |

a: Twelve species are phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene, and benzo(ghi)perylene.

b: The amount of eluted nicotine from the sample was shown.

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Table 1 Concentrations of metals and PAHs in road dust \(^\text{25)}\).

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synthetic moderately hard water, and was mixed with the reference sediment 24 h prior to start of the test. The exposure was performed using 80 organisms for the control and 160 organisms for the zinc treatment. After six days of exposure at 25°C in darkness, all surviving ostracods (58 organisms for the control and 103 organisms for the zinc treatment) were used for RNA extraction and transcriptome sequencing.

b) Road dust exposure

To determine the sub-chronic toxicity of urban road dust, road dust exposure was also performed according to ISO 14371 methods[14]. The exposure was initiated by transferring 10 hatched ostracods to each well of a six-well plastic microplate containing a mixture of 1 mL urban road dust diluted with reference sediment, 2 mL synthetic moderately hard water, and 2 mL green alga suspension. The synthetic moderately hard water and urban road dust were mixed 24 h prior to start of the test. The two-fold dilution series were prepared, consisting of 6.25%, 12.5%, and 25% (dry, v/v) of urban road dust. The exposure was separately performed for 12.5% and 25% (Run-1), and for 6.25% (Run-2). For each run, the negative control using reference sediment was tested. Each concentration consisted of six replicates with 10 organisms per replicate. The number of surviving organisms was counted every day during the exposure. At the start and end of exposure, the length of surviving organisms was recorded. The length was measured using Image J (version 1.48; National Institutes of Health) based on photos taken by a stereomicroscope (SZX12, Olympus) with a DP12 digital camera (Olympus). pH and conductivity in the overlying water were measured at the start and end of exposure using a pH meter (LAQUAtwin, Horiba) and a conductivity meter (B-173, Horiba).

For qPCR experiments, road dust exposure was performed at a concentration of 6.25%, as described above. Each treatment consisted of three replicates with 30 organisms per replicate. After one, two, and six days of exposure, surviving ostracods were collected and preserved in RNAlater™ (Ambion) until RNA extraction.

(3) RNA isolation and sequencing

Total RNA was extracted from ostracods preserved in RNAlater™ using QIAGEN RNeasy Mini Kit (Qiagen), after homogenization with a plastic pestle. RNA integrity was checked using Agilent Bioanalyzer (Agilent Technologies). Since the 28S rRNA of many crustacean species easily breaks into small fragments[29,30], RNA integrity was checked not by RIN (RNA Integrity Number), but by the presence of clear 18 rRNA band and the absence of broad smears below the 18 rRNA band (Fig. A1). RNA purity was verified at A260/A280 ratios greater than 1.8 using Nanodrop™ 1000 (Thermo Fisher Scientific). RNA amount was quantified by Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) and Qubit® RNA HS Assay Kit.

A cDNA library was constructed using TruSeq RNA Sample Prep Kit v2 (Illumina) following the manufacturer’s protocols, and sequenced by HiSeq 2500 (101 bp, paired-end). The obtained raw reads were deposited on the Sequence Read Archive (accession No.: DRX120131 and DRX120132).

After the removal of the adapter sequences using Trimmomatic (ver 0.36)[31], the preprocessing of reads was performed on DNA Data Bank of Japan (DDBJ) Read Annotation Pipeline (expired in February 2019)[32]. First, low-quality bases with Phred scores below 19 were trimmed from the 5’ and 3’ ends, and trimmed reads with a length under 24 bases were discarded. Second, trimmed reads, which included more than 30% of bases with Phred scores below 14, were removed. When one read of the pair was removed, the other read of the pair was also discarded. The remaining reads were de novo assembled by Trinity[33] (ver. 2.1.1) with the default setting on DDBJ Read Annotation Pipeline, and deposited on Transcriptome Shotgun Assembly (accession No.: ICLE01000001 to ICLE01285314). The assembled genes were annotated against the Swissprot database (557,491 sequences; downloaded on 21 May 2018) using BLAST+ (ver. 2.5.0) with an E-value cut-off of 10^{-10}. Based on the annotation results, antioxidant genes were identified and their open reading frames (ORFs) were deduced using ORFFinder at the NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/).

(4) qPCR

Total RNA was extracted from surviving ostracods (up to 30 individuals per replicate) according to the methods described above. Total RNA of 95.9 ng to 809.0 ng was reverse-transcribed to cDNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara) and oligo-dT primer after DNase treatment using DNA-free™ (Ambion). qPCR analyses were performed in 20 µL reactions containing 5 µL of tenfold diluted cDNA, 10 µL of Light Cycler® 480 SYBR Green I Master (Roche Diagnostics), and 0.5 µM primers (Table 2; four target and one reference genes) on the Light Cycler 480 Real-Time PCR System (Roche Diagnostics). PCR was conducted with 5 min incubation at 95°C, followed by 45 cycles of 30 s denaturation at 95°C, 30 s annealing at 59°C, and 60 s extension at 72°C. The gene-specific primers were designed based on the de novo assembled sequences using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer dimers and the specificity of amplified products were checked by melting curve analyses and agarose gel
electrophoresis. The Cp (crossing point) values were determined using the second derivative maximum method by the LightCycler Software. For all samples and genes, the difference in Cp values between the cDNA sample and no-reverse transcription control was more than five cycles, indicating that the contribution of genomic DNA contamination was trivial. The relative expressions of target genes were calculated by the 2^ΔCp methods using Cp values of 60S acidic ribosomal protein P0 (rplp0) for normalization.

(5) Statistical analysis
All statistical analyses were performed in R software (ver. 3.5.0). Statistically significant differences in ΔCp values between the control and road dust exposure treatments at each time point were tested by a two-tailed Welch’s t-test using the R stats package. Ostracod mortality data were modeled using a generalized linear model (GLM) with a binomial error distribution and log logit link, and differences in mortality between treatments were determined using a likelihood ratio test with chi-square approximation (using the R anova function). The 50% lethal concentration (LC50) was calculated based on the median lethal time (LT50) was computed using LT_logit function in the ecotox R package (ver. 1.4.0). A value of p < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

(1) Transcriptome of *H. incongruens*

The transcriptome library was constructed from the freshwater ostracod *H. incongruens* using high-throughput sequencing (Table 3). A total number of 266,794,398 raw reads was obtained from paired-end reads with Q20 of 97.0% and GC content of 50.6%. By using Trinity software, a total of 285,314 assembled reads were obtained with an N50 of 1,050 nucleotides (nt). Among them, 75,570 reads (26.5%) were annotated against the Swissprot.

(2) Stress response genes

Based on the annotation against the Swissprot, four antioxidant genes (*cat, gpx, gstcd*, and *sod*) and a reference gene (*rplp0*) with stable expression levels across various environmental conditions were identified. The results of homology search using BLASTx program indicated that the nucleotide sequences of these genes shared high identity with those of other species (Table 4). The sequence of *cat* showed high identity with those of other species, such as a decapod *Palaemon carinicauda* (82%), a decapod *Penaeus japonicus* (80%), a copepod *Paracyclops nana* (80%), and a fruit fly *Drosophila melanogaster* (77%). The sequence of *gpx* showed high identity with those of other species including a whale shark *Rhincodon typus* (64%), a wild boar *Sus scrofa* (62%), and a lamprey *Lethenteron camtschaticum* (62%). The sequence of *gstcd* showed high identity with those of other species such as a tick *Ixodes scapularis* (77%), *B. germanica* (73%), and *D. melanogaster* (66%).

Except *cat* and *rplp0*, the assembled sequences contained the complete ORFs. The lengths of estimated ORFs were 426 nt (*gpx*), 1,899 nt (*gstcd*), and 471 nt (*sod*). The ORFs encoded 141 amino acids (aa) (*gpx*), 632 aa (*gstcd*), and 156 aa (*sod*).

The deduced amino acid sequence of *cat* contained partial catalase-like heme-binding proteins and protein domains (cl09506) and a conserved active site motif FDRERIPERVVHAKGAG. The amino acid sequence analysis showed that *gpx* had the domain of thioredoxin-like superfamily (cl00388) and contained a conserved active site motif WNFKEKF. The sequence analysis also showed that *gstcd* contained S-
Table 4 List of stress response and reference genes with their homologs.

| Accession No. | Sequence length (nt) | Homologous protein | Identity (%) | Alignment length (aa) |
|---------------|---------------------|--------------------|--------------|----------------------|
| cat           | ICLE01123846        | 723                | P17336: Catalase, Drosophila melanogaster | 77.0 | 191 |
| gpx           | ICLE01080538        | 1,254              | Q8MJ14: Glutathione peroxidase 1, Sus scrofa | 62.4 | 140 |
| gstcd         | ICLE01229961        | 2,663              | Q9VJ34: Glutathione S-transferase C-terminal domain-containing protein homolog, Drosophila melanogaster | 35.5 | 543 |
| sod           | ICLE01199681        | 620                | P10791: Superoxide dismutase [Cu-Zn], Drosophila virilis | 70.2 | 151 |
| rplp0         | ICLE01203318        | 405                | P19889: 60S acidic ribosomal protein P0, Drosophila melanogaster | 65.8 | 120 |

adenosylmethionine-dependent methyltransferases domain (cl17173), and that rplp0 had the domain of rplP0 super family (cl25391). The deduced amino acid sequence of sod contained copper/zinc superoxide dismutase domain (pfam00080), copper binding sites (His 48, 50, 65, and 122), and zinc binding sites (His 65, 73, 82, and Asp 85). These results indicated that the obtained genes contained the conserved protein domains and might have functions related to antioxidant reactions as reported in other species.

(3) Toxicity of urban road dust

During the exposure tests, pH ranged from 7.6 to 9.0, and conductivity ranged from 0.98 to 2.90 (Table 5). At the end of exposure, the survival rates in the control were 95.0 ± 8.4% and 90.0 ± 6.3% (Mean ± SD) for Run-1 and Run-2 (Table 5). Both the survival rates met the criterion of > 80% recommended by ISO methods14). The mean lengths of survival ostracods in the control after six days were 729.0 ± 17.3 mm (Run-1) and 945.2 ± 20.3 mm (Run-2). These values were > 1.5 times larger than the mean length at the start of exposure, which met the criterion set by ISO methods14).

When the data of Run-1 and Run-2 were combined, six-day LC20 and LC50 were calculated to be 5.04% (95% confidence interval (CI): 4.24–5.85%) and 6.85% (95% CI: 6.14–7.56%) (Fig. 1a). These results indicate that the road dust sample required a dilution of approximately 20 times to become non-toxic (< 20% mortality). Such dilution may sometimes not be achieved in urban areas, as the mass contribution of road dust to urban river sediment was estimated to be 0.8% to 6.8%38). Ten-day LC50 of the road dust sample used in this study was 37.9% for an estuarine amphipod Grandidierella japonica25), indicating that the six-day ostracod test was more sensitive to the road dust sample than the amphipod test.

Time course survival rates of H. incongruens exposed to urban road dust are shown in Fig. 1b. LT50 was computed to be > 6 days, 1.57 (CI: 1.11–1.96) days, and 0.95 (CI: 0.79–1.10) days at concentrations of 6.25%, 12.5%, and 25%, respectively. Whereas the deaths of the ostracods occurred rapidly at higher concentrations such as 12.5% and 25%, they occurred gradually at 6.25% of urban road dust.

(4) Expression changes by urban road dust

The survival rates in the exposure tests for qPCR experiments were greater than 90% at every timing in the control, and were 82.2 ± 10.9%, 77.8 ± 22.2%, and 41.1 ± 26.7% (Mean ± SD) in the road dust treatment after one, two, and six days, respectively. While survival rates were lower than those obtained in the
dose-response test (Fig. 1), statistically significant differences in survival rates were not found ($p = 0.07$, likelihood ratio test).

The relative expression levels of antioxidant genes in *H. incongruens* exposed to urban road dust for one, two, and six days are shown in Fig. 2. All the tested genes did not show statistically significant expression changes for either exposure period ($p > 0.25$). Their absolute fold changes were less than 2, with the exception of 3.47-fold of *gpx* at Day 2. These genes play roles in the antioxidant defense systems through converting superoxide radicals to H$_2$O$_2$ (by *sod*) and detoxifying H$_2$O$_2$ (by *cat* and *gpx*). Their expressions in aquatic species are some of the most frequently reported effects of road runoff\(^3\) and related contaminants, such as metals\(^4\) and hydrocarbons\(^2\),\(^4\). For example, *gpx* was upregulated in an amphipod *G. japonica* after 96-h exposure to zinc\(^4\). The expression of *sod* in a water flea *Daphnia magna* was increased after 48-h exposure to copper\(^4\). These previous reports seemed not to be consistent with our results, where no statistically significant changes were observed despite the high lethal toxicity and high concentrations of metals (Table 1). This was possibly due to few replicates (n=3) and low statistical power in this study. Another possible reason was that oxidative stress was not a major mode of action of the road dust toxicity tested in this study. As the chemical characteristics and toxicity of road dust are site-specific\(^1\), other types of stress might be dominant in the tested dust. Though high concentrations of metals were detected in the tested dust, the bioavailability of metals was unclear. For example, as copper predominantly exists as organic complexes (49%–58%) and carbonates (40%–49%) in road dust leachate\(^3\), the majority of copper might not contribute to road dust toxicity. Therefore, high concentrations of metals in the dust were not contradictory to no apparent changes in the expressions of antioxidant genes. In fact, our previous study using comprehensive RNA sequencing analyses suggested that antioxidant reactions were not dominant in an amphipod *G. japonica* exposed to urban road dust\(^4\). This study tested limited types of antioxidant genes and road dust samples, therefore we cannot conclude that road dust did not induce oxidative stress in *H. incongruens*. Further research is required to investigate changes in a broad set of antioxidant genes, such as glutathione reductase and peroxiredoxin, in response to several types of road dust samples.

As demonstrated by the sequence analysis, the identified antioxidant genes are reliable candidates for diagnostic and early-warning biomarkers of oxidative stress. However, in this study, we did not verify the differential expression of the identified genes in response to oxidative stress. The identified genes need further verification using various oxidative chemicals.

### 4. CONCLUSIONS

The comprehensive RNA sequences of the benthic ostracod *H. incongruens* were determined using a high-throughput sequencing technology. From the determined sequences, antioxidant genes such as *cat* and *gpx* were identified. The changes in their expressions in response to urban road dust were investigated by using qPCR; however, there were no statistically
Fig A1 Total RNA electropherogram of *Heterocypris incongruens* by Bioanalyzer. a–f) Samples after one day; g–l) Samples after two days; m–r) Samples after six days. X axis represents the length of nucleotide, and Y axis represents fluorescence unit.
significant changes. Although further verification of the identified genes is needed, our transcriptome data will become useful resources for future ecotoxicological studies and environmental monitoring using this species.

**AUTHOR CONTRIBUTIONS:** All authors conceived and designed the study. MT conducted the experiments. MT and KH analyzed the data. KH wrote the manuscript.

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**APPENDIX**

Electropherograms of total RNA obtained from the ostracods are shown in Fig. A1.

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