Aquatic Toxicology 201 (2018) 73–82

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Genome-wide identification of 99 autophagy-related (*Atg*) genes in the monogonont rotifer *Brachionus* spp. and transcriptional modulation in response to cadmium

Hye-Min Kang[a], Jin-Sol Lee[a], Min-Sub Kim[a], Young Hwan Lee[a], Jee-Hyun Jung[b], Atsushi Hagiwara[c,d], Bingsheng Zhou[e], Jae-Seong Lee[a,*], Chang-Bum Jeong[a,*]

[a] Department of Biological Science, College of Science, Sungkyunkwan University, Suwon 16419, Republic of Korea
[b] Oil & POPs Research Group, Korea Institute of Ocean Science & Technology, Geoje 53201, Republic of Korea
[c] Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki 852-8521, Japan
[d] Organization for Marine Science and Technology, Nagasaki University, Nagasaki 852-8521, Japan
[e] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

ARTICLE INFO

Keywords:
Autophagy
Autophagy-related genes
Atg
Rotifer
Brachionus spp.

ABSTRACT

Autophagy originated from the common ancestor of all life forms, and its function is highly conserved from yeast to humans. Autophagy plays a key role in various fundamental biological processes including defense, and has developed through serial interactions of multiple gene sets referred to as autophagy-related (*Atg*) genes. Despite their significance in metazoan life and evolution, few studies have been conducted to identify these genes in aquatic invertebrates. In this study, we identified whole *Atg* genes in four *Brachionus* rotifer spp., namely *B. calyciflorus*, *B. koreanus*, *B. plicatilis*, and *B. rotundiformis*, through searches of their entire genomes; and we annotated them according to the yeast nomenclature. Twenty-four genes orthologous to yeast genes were present in all of the *Brachionus* spp. while three additional gene duplicates were identified in the genome of *B. koreanus*, indicating that these genes had diversified during the speciation. Also, their transcriptional responses to cadmium exposure indicated regulation by cadmium-induced oxidative-stress–related signaling pathways. This study provides valuable information on 99 conserved *Atg* genes involved in autophagosome formation in *Brachionus* spp., with transcriptional modulation in response to cadmium, in the context of the role of autophagy in the damage response.

1. Introduction

Autophagy is a key cellular homeostasis system that functions as a cell survival and death mechanism by degrading misfolded proteins and cytoplasmic organelles (Klionsky and Emr, 2000). Autophagy regulates cellular homeostasis in response to various stimuli when cell damage is reversible; otherwise, it regulates cell death. Autophagy is induced by various stimuli including environmental stressors, and also plays a crucial role in cellular remodeling during organism development (Penaloza et al., 2006; Di Bartolomeo et al., 2010). To date, most of the ecotoxicological studies have focused on apoptosis, despite the intermediate role of autophagy in cell-death and cell-recycling systems in response to environmental stressors (Codogno and Meijer, 2005). Thus, autophagy plays an important role in fundamental biological processes, including defense, in response to toxicants. In invertebrates, autophagy is involved in the early development of the sea urchin *Paracentrotus lividus* (Agnello et al., 2016), and is also part of the defense system that responds to cadmium (Chiarelli et al., 2011). In the rotifer *Brachionus koreanus*, autophagy *via* the p38 mitogen-activated protein kinase (MAPK) signaling pathway is stimulated in response to cadmium-induced oxidative stress (Kang et al., 2018).

Genes involved in these processes are termed autophagy-related (*ATG*) genes. Among these, the essential genes for autophagosome formation are referred to as the core machinery genes. They are classified into three subgroups (Yang and Kilonsky, 2011; Xie and Klionsky, 2007): Atg9 and its cycling system, ubiquitin-like protein conjugation systems, and the phosphoinositide-3-kinase (PI3K) complex. The ATG genes were first highlighted in yeast with the identification and characterization of related genes (Klionsky et al., 2003). Similar studies then investigated *Caenorhabditis elegans* and other eukaryotes, and further investigated yeast (Klionsky et al., 2003; Meléndez and Neufeld, 2008).

However, although invertebrate genomes have become more

* Corresponding authors.
E-mail addresses: jslee2@skku.edu (J.-S. Lee), cbjeong3@skku.edu (C.-B. Jeong).

https://doi.org/10.1016/j.aquatox.2018.05.021
Received 25 April 2018; Received in revised form 24 May 2018; Accepted 26 May 2018
Available online 30 May 2018
0166-445X/

accessible with advances in sequencing technologies, there is still a huge gap in our knowledge about the conservation and evolution of invertebrate *Atg* genes. In our study, we focused on the identification of *Atg* genes in four *Brachionus* spp., *B. plicatilis*, *B. rotundiformis*, *B. koreanus* and *B. calyciflorus,* with further genomic analysis using their genome and RNA-seq databases, which have been established recently (unpublished data; Lee et al., 2011, 2015; Gribble and Mark Welch, 2017; Kim et al., 2017, 2018). Furthermore, these species are distributed worldwide in different environmental conditions (Mills et al., 2017) due to species-specific optimal habitat condition. Thus, comparative analysis of *Brachionus* spp. would improve our understanding of the speciation mechanism and of the evolutionary relationships of species within the same genus.

Rotifers are ubiquitous in the aquatic environment. They play a crucial role in the ecosystem as a primary consumer by bridging the energy flow between producers and consumers (Snell and Janssen, 1995; Dahms et al., 2011). Also, rotifers provide many advantages for laboratory studies, with their small size (≈150–250 μm), short life cycle (≈24 h), high fecundity, genetic homogeneity, and easy maintenance in laboratory conditions (Snell and Janssen, 1995; Dahms et al., 2011). Furthermore, the RNA-seq and the genome databases of several *Brachionus* spp. have been well established (Lee et al., 2011, 2015; Gribble and Mark Welch, 2017; Kim et al., 2017, 2018), strengthening rotifers as a useful model for various research fields, including ecophysiology and ecotoxicology.

In this study, we aimed to identify core machinery *Atg* genes in the four *Brachionus* rotifer spp. *B. plicatilis*, *B. rotundiformis*, *B. koreanus*, and *B. calyciflorus,* and to characterize their transcriptional modulation patterns in response to cadmium. Our genome-wide comparative analysis of *Atg* genes in these species will expand our knowledge of autophagy systems and their role in defense in invertebrates. It will also expand our mechanistic understanding of the evolution of defense among the rotifer species.

2. Materials and methods

2.1. Rotifer culture

The monogonont rotifer *B. koreanus* was collected in Uljin (36°58′43.01″N, 129°24′28.40″E), in South Korea. *Brachionus plicatilis*, *B. rotundiformis*, and *B. calyciflorus* (originally collected in Zwartenhoek in the Netherlands [52°02′63″N and 4°18′35.5″E] in a resting egg state by Dr. Steven A.J. Declerck) were kindly provided by Prof. Atsushi Hagiwara (Nagasaki University, Nagasaki, Japan). A single rotifer from each species was isolated, cloned parthenogenetically, and maintained in filtered artificial seawater (ASW) (Tetra Marine Salt Pro, Tetra, Cincinnati, OH, USA). The strain was maintained by serial transfer of asexual populations at 25 °C under a light:dark 12:12 h photoperiod with practical salinity units (psu) of salinity as follows: 22 psu for *B. plicatilis*, 15 psu for *B. rotundiformis* and *B. koreanus*, and freshwater for *B. calyciflorus*. The green alga *Chlorella* sp. was used as a live diet (approximately 6×10^6 cells/mL). Species identification was confirmed by morphological analysis and sequencing of the mitochondrial DNA gene cytochrome oxidase 1 (*CO1*) as described previously (Hwang et al., 2013, 2014; Mills et al., 2017).

2.2. Identification of Atg genes

A genomic DNA sample from each *Brachionus* sp. was sequenced using the Illumina HiSeq 2000 platform (300 bp, 500 bp, and 800 bp as paired-end libraries and 2 kb, 5 kb, and 10 kb as mate-pair libraries) at the National Instrumentation Center for Environment Management (NICEM), Seoul National University in Seoul, South Korea. After sequencing, we pre-processed the raw sequenced reads using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic). *De novo* assembly of pre-processed raw reads was performed using the Platanas assembler v1.2.4 (http://platanus.bio.titech.ac.jp) and HaploMerger 2 v20151124 (http://mosas.sysu.edu.cn/genome/download_softwares.php). Whole genome assembly yielded a total genome length of 85,731,059 (scaffold nos. 567; N50 = 1.86 Mb), 106,939,251 (scaffold nos. 716; N50 = 1.16 Mb), 57,713,393 (scaffold nos. 216; N50 = 2.69 Mb), and 129,636,934 bp (scaffold nos. 1,041; N50 = 3.65 Mb) in *B. koreanus*, *B. plicatilis*, *B. rotundiformis* (unpublished data), and *B. calyciflorus* (Kim et al., 2018), respectively.

To obtain genes encoding *ATGs*, a TBLASTN search (E-value of 10^{-5}) was performed on the genome sequence assembly of *Brachionus* spp. (unpublished data; Kim et al., 2018), using yeast *Atg* genes as queries. All acquired contigs were mapped to the genome for obtaining the complete DNA sequence, using Exonerate (http://www.ebi.ac.uk/about/vertebrate-genomics/software/exonerate). Annotation and nomenclature of all *Atg* genes were completed based on amino acid similarities and phylogenetic analysis. All gene information was registered to the GenBank database, and the accession numbers of each gene are appended in Tables 1 and S1.

2.3. Gene structure and phylogenetic analyses

Exon/intron structures and translation initiation/termination sites for *Atg* genes in *Brachionus* spp. were analyzed by mapping corresponding cDNA sequences on the full-length genomic copies using Geneious software (Ver. 8.1.9; Biomatters Ltd., Auckland, New Zealand).

To investigate the evolutionary relationships of the *Brachionus* spp. Atg genes, all the Atg genes identified in *B. plicatilis*, *B. rotundiformis*, *B. koreanus*, and *B. calyciflorus* were subjected to phylogenetic analysis, along with *Homo sapiens* ATG genes. The amino acid sequences of the Atg genes from *Brachionus* and *H. sapiens* were then aligned using MEGA software (ver. 7.0; Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA) with the ClustalW alignment algorithm, as described previously (Thompson et al., 1997; Kumar et al., 2016). To establish the best-fit substitution model for phylogenetic analysis, the model with the lowest Bayesian Information Criterion (Schwarz, 1978) and Akaike Information Criterion (Hurvich and Tsai, 1989; Posada and Buckley, 2004) scores was estimated using a maximum likelihood (ML) analysis. Using the results of the model test, maximum likelihood phylogenetic analyses were performed using RAxML (ver. 8.2.8) (Stamatakis et al., 2005) with the LG + G + I model.

2.4. Cadmium exposure, total RNA extraction, and quantitative real-time PCR

Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$; molecular weight 228.36, Sigma-Aldrich, St. Louis, MO, USA; purity > 98%) was used for exposure. Approximately 2000 individuals of adult rotifers (> 150 μm) were exposed to various concentrations of cadmium (control, 10 [43.79 μM], 20 [87.58 μM], and 30 [131.37 μM] mg/L) for 6 h in 50 mL ASW. Exposure time was chosen according to our previous study, as autophagosomes were strongly detected at this time point in the rotifer *B. koreanus* (Kang et al., 2018). Rotifers were not fed during exposure. All experiments were performed in triplicate.

Total RNA was then extracted from whole bodies of rotifers (≈2000) by using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and stored at −80 °C until use. Spectrometric analysis (QIAxpert®, QIAGEN GmbH, Hilden, Germany) was performed at 230, 260, and 280 nm to measure the quality and quantity of the extracted total RNA. Single-stranded cDNA was synthesized from 1 μg of total RNA from each sample using a commercially available kit (SuperScript™ III Reverse Transcriptase kit, Invitrogen).

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using target-gene–specific forward and reverse primers under the following conditions: 95 °C/4 min; 35 cycles of 94 °C/30 s,