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Light-dependent transcriptional events during resting egg hatching of the rotifer *Brachionus manjavacas*

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ABSTRACT

Rotifer resting eggs often have to endure harsh environmental conditions during the diapause phase. They are stimulated by light to hatch. In order to study the hatching mechanism, we observed resting eggs and measured their transcriptional expression under different light exposure periods (total darkness, and after 30 min, and 4 h light). By using differential-display reverse transcription PCR (DDRT-PCR), we isolated 80 genes that displayed different expression patterns in response to the three light treatments: 20 genes were expressed in total darkness, 40 different genes were differentially expressed under 30 min light, and 20 further genes were expressed after 4 h of light. The resting eggs showed no phenotypic differences in embryonic development during the 4 h illumination period. In general, the expression patterns of the analyzed genes in resting eggs were differentially modulated by light exposure time. In total darkness, resting eggs mainly expressed genes encoding cell defense and homeostasis functions. In the 30 min illumination group, we found enriched expression of genes encoding fatty acid metabolism-related components, including Acyl-CoA dehydrogenase (*ACAD*). Genes encoding cellular and embryonic developmental functions were highly observed in the 30 min-illuminated group but were not observed in the 4 h-illuminated group. Real-time RT-PCR revealed that several transcripts such as encoding V-type H(+)-translocating pyrophosphatase (*V-PPase*) and *Meckelin* had prolonged expression levels when exposed to light for 4 h. In the 4 h illuminated group, the RecQ protein-like 5 *(RECQL5)* gene was enriched. This *RECQL5* gene may be expressed to protect the developing embryo from continuous light exposure. The data presented in this study indicate that DDRT-PCR-aided gene screening can be helpful to isolate candidate genes involved in the hatching process.

1. Introduction

Under stressful conditions such as extremes in temperature, or inadequate nutritional resources, some animals migrate away, while others modify their behaviors and/or habitats to reduce environmental stress. Dormancy is another adaptive mechanism to minimize the impact of stressors and to protect organisms from harsh environmental conditions (Lubzens et al., 2010). Dormancy includes diapause and quiescence that are distinguished from a queue of endogenous or exogenous control, respectively (Ricci, 2001). During dormancy, organisms' physiological and metabolic activities (including growth, development and reproduction) temporarily cease in order to conserve energy for the following active season. In the hydrosphere, planktonic metazoa such as monogonont rotifer, *Artemia*, copepods and cladocera have the ability to become dormant by entering the resting egg (cyst) stage (Dahms, 1995). Among those microscopic aquatic invertebrates, rotifers frequently used as model laboratory organism because of their small size, simple organization, quick growth (<24 h) and ease of mass cultivation (Dahms et al., 2011). In addition, the dormancy mechanism has been more clearly described in rotifers than in other species (Gilbert, 2003). During rotifer dormancy, two phenomena may occur, called diapause and quiescence (Ricci, 2001).

Rotifers have a heterogenic life cycle that includes both sexual (mictic) and asexual (amictic) reproduction (Snell, 1987; Hagiwara et al., 1989; Gilbert, 2003). The asexual reproduction dominates and amictic females parthenogenetically produce exclusively female offspring. Sexual reproduction in rotifers is initiated by environmental cues known as mixis inducers. Mixis inducers include both external factors (e.g. population density, temperature, salinity, food, juvenile hormone, and serotonin) and internal factors (e.g. genetic variation, culture history, cumulative generation, and aging) (Snell, 1987; Gallardo et al., 2000; Gilbert, 2003). Once sexual reproduction is triggered, mictic females undergo meiosis, which yields haploid gametes. If a male fertilizes these gametes, diploid resting eggs result. The resulting resting eggs are remarkably tolerant to unfavorable conditions

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and can retain viability for decades (reviewed in Gilbert, 2004 and Hagiwara et al., 2007). A prior study suggested that several genes are associated with dormancy and desiccation tolerance in the monogonont rotifer *Brachionus plicatilis* (Denekamp et al., 2009). Despite these data, the underlying molecular mechanism and gene profile of dormancy and resting egg hatching in Rotifera are still incompletely understood.

Many researchers have extensively studied the environmental conditions, including light, temperature and salinity, that may affect resting egg hatching (Hagiwara et al., 1989, 1995; Hagiwara and Hirayama, 1993; Hagiwara, 1994). It is currently well known that in favorable environments, light stimulation triggers resting eggs to hatch as amictic females (Gilbert, 1974, 2007; Pourriot and Snell, 1983; Hagiwara and Hino, 1989; Wallace et al., 2006). Rotifer resting eggs, in particular, cannot initiate embryonic development without light. The eggs will not hatch in the absence of light, even if they are incubated under optimal conditions for hatching. Previously, it was thought that light triggered hatching via light-induced peroxide production in seawater and fatty acid oxidation to prostaglandins (E_1, E_2 or $F_{2\alpha}$) inside of the embryo. These hypotheses were supported by the fact that both hydrogen peroxide and prostaglandins can trigger resting egg hatching, even in darkness (Hagiwara et al., 1995). However, empirical studies have yet to substantiate these hypotheses.

Our objective was to better define the mechanism between diapause and hatching. In order to do so, we measured the transcription expression of resting monogonont rotifer *Brachionus manjavacas* eggs under different periods of light irradiation (total darkness, 30 min, and 4 h) using differential-display reverse transcription PCR (DDRT-PCR). This method has been used previously to isolate differentially enriched genes in response to various stressors in a short time period (Callejas and Gutiérrez, 2003; Medini et al., 2009; Siquieroli et al., 2009). The results were validated using real-time RT-PCR. Finally, we determined the putative function of each transcript expressed during embryonic development using bioinformatics-aided characterization of the enriched genes. This paper provides new insight into the light-mediated molecular hatching process in the rotifer resting egg.

2. Materials and methods

2.1. Resting egg preparation

This study employed the Australian stain of *B. manjavacas,* which demonstrates active induction of sexual reproduction (Araujo and Hagiwara, 2005; Kim and Hagiwara, 2011). In order to produce a sufficient number of resting eggs, the rotifers were cultured at 25 °C in 40 L of 11 parts per thousand (ppt) artificial seawater (Hagiwara et al., 1989; Hagiwara and Lee, 1991). These conditions are optimal for producing resting eggs. The rotifers were fed daily with a mixture of *Tetraselmis tetrathele* (0.12×10^6 cells/mL) and *Nannochloropsis oculata* (3.5×10^6 cells/mL). Two rotifer diets were independently cultured at 25 °C in 100 L of 11 ppt artificial seawater with continuous fluorescent light (2000 l×) and were mixed immediately before feeding. The rotifers were cultured for 12 days and the resting eggs were harvested on the last day. The harvested resting eggs were preserved in a 4 °C refrigerator (in total darkness) for four months. Prior to sample preparation, the hatchability of the preserved resting eggs was confirmed. The threshold light condition to induce hatching was 4400 l× for 30 min. In addition, illumination for 4 h had no effect on embryonic development with regard to morphological traits (unpublished data). Fifty resting eggs were randomly selected from the preserved stock. These eggs were transferred into a 6-well polystyrene microplate, incubated for 24 h under continuous fluorescent light (3000 l×), and maintained under the same conditions as used to form the resting eggs (25 °C, 11 ppt). Three 20 W white fluorescent bulbs (FL20SW, Toshiba, Japan) in the incubator were used to irradiate these 50 eggs at 3000 l×. A mean of six replicates was used to calculate the hatching rate.

2.2. Morphological observation

The preserved resting eggs (n ~ 18,000 eggs) were transferred into 500 mL glass beakers (from 4 to 25 °C) containing 200 mL of 11 ppt diluted natural seawater. The three prepared beakers were incubated at 25 °C under the following illuminance conditions; in total darkness (0 min, control) and 3000 l× fluorescent light for 30 min and 4 h. After light illumination, the morphological characteristics including diameter (width and length) and cell division of the resting eggs were compared between the control and the 4 h treatment groups using microscopic observation.

2.3. RNA extraction and cDNA synthesis

The light-treated (0, 30 min, and 4 h) resting eggs were rinsed several times with milli-Q water (millipore 0.24 μm) to remove saline water and feces. Total RNA was extracted with ISOGEN (Nippon gene, Tokyo, Japan) based on the supplied protocol and was suspended in an appropriate volume of autoclaved milli-Q water. RNAqueous® (Small Scale Phenol-Free Total RNA Isolation Kit, Ambion®, Tokyo, Japan) was used to recover the total RNA with enzyme reactions according to the instruction manual. The total amount of isolated RNA was quantified using UV spectrophotometry (Gene Spec III, Naka instruments Co., Ltd, Ibaraki, Japan). Total RNA isolated from some tissues often contains relatively high levels of genomic DNA. Therefore, 5 μg of total RNA in each sample was treated with TURBO DNA-free™ (Ambion®, Carlsbad, CA, USA) in order to remove the genomic DNA and to subsequently remove DNase I and divalent cations from the samples. cDNA was synthesized with 0.1 μg of isolated RNA using Advantage® RT-for PCR kit (Clontech, Tokyo, Japan) with the following ingredient mixture in a 500 μL reaction tube: RNA, oligo$(dT)_{18}$ primer, milli-Q water and mineral oil to prevent evaporation. These mixtures were incubated at 70 °C for 3 min. Next, a 5× reaction buffer, dNTP mix (10 mM each) and MMCV-RT reverse transcriptase were added to the tubes. Reverse transcription was performed at 42 °C for an hour. When this process was finished, the reaction mixture was heated to 75 °C for 10 min to inactivate the reverse transcriptase. The synthesized cDNA was kept at −20 °C until use.

2.4. Differential display reverse transcription-PCR (DDRT-PCR)

DDRT-PCR analysis was performed using the Delata™ Differential Display Kit (Clontech, Tokyo, Japan). The total volume of the PCR reaction (20 μL) contained the following ingredients: 1 μL cDNA template, 0.4 μL 50× dNTP mix, 2 μL 10× KlenTaq PCR reaction buffer, 1 μL 20 pmol/μL of T-primer, 1 μL 20 pmol/μL of P-primer, 0.4 μL 50× Advantage KlenTaq Polymerase and 14.2 μL Milli-Q water. The PCR cycle included one cycle at 94 °C for 5 min, 40 °C for 5 min, 68 °C for 5 min; two cycles at 94 °C for 30 s, 40 °C for 30 s, and 68 °C for 5 min; 30 cycles at 94 °C for 20 s, 60 °C for 30 s, 68 °C for 2 min; and one cycle at 68 °C for 7 min. The 30 cycles of synthesis were carried out with an annealing temperature of 60 °C. The amplified cDNA in the PCR products was separated using agarose gel electrophoresis (2.0% agarose gel, Metaphor® Agarose, Cambrex, Rockland, USA). The gels were stained with SYBR Green (Molecular Probes Inc., Invitrogen, Carlsbad, CA, USA) for 30 min and photos were taken to compare the gene transcription among different treatments. The differentially expressed gene fragments were excised from the gel and preserved at −20 °C.

2.5. Reamplification and purification

The excised gene fragments containing cDNA fragments were melted and crushed with a micro-pipette; 1 μL of paste was extracted. Reamplification was performed in the same way as was the prior amplification. Eighty reamplified cDNA fragments were electrophoresed on