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Visualization of ecdysteroid activity using a reporter gene in the crustacean, *Daphnia*

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ABSTRACT

Ecdysone is a hormone known to play a pivotal role in crustaceans and insects. In order to evaluate the ecdysone activities in the environment and within the organism, we have developed a biomonitoring *Daphnia* strain by introducing a reporter gene. In this study, the ecdysone response element was inserted in the upstream region of a reporter gene, and the DNA construct was injected into *Daphnia* eggs. The expression of the reporter gene was detected during the early embryonic development stage. In addition, when the eggs expressing the reporter gene were exposed to ecdysone, there was enhanced expression of the reporter gene at detectable levels, while the presence of an antagonist led to its downregulation. These results suggested that this system could be potentially developed for monitoring ecdysone activities in media.

1. Introduction

The subphylum Crustacea comprises many marine organisms, including crabs, lobsters, shrimp, and krill, and its economic and ecological importance is widely recognized. Crustaceans have quite different hormonal systems from vertebrates and the two major classes of crustacean hormones, ecdysteroids and terpenoids, are known to regulate various aspects such as growth, reproduction, and metabolism. The pivotal role of ecdysteroids has been well described in insects, in which they trigger signals for changing cell shape, proliferation, and cell death in imaginal discs. In addition, ecdysteroids are known to play essential roles during embryonic development, which has been validated in insects (Kozlova and Thummel, 2003). Although it is believed that ecdysteroids control a variety of physiologically important events, the shared and different functions of ecdysteroids between crustaceans and insects are not well understood.

Recently, it was reported that chemicals that interfere with ecdysteroid signaling potentially cause adverse effects in crustaceans (LeBlanc, 2007). Chemicals having estrogen receptor agonist activity have the potential to bind to ecdysone receptors (Dinan et al., 2001), and an agricultural fungicide, fenarimol, has anti-ecdysteroidal activity (Mu and LeBlanc, 2002). As several chemicals contaminating the environment may possess hormonal activities, it is important to develop a monitoring system to evaluate the chemical impacts on crustaceans. In order to monitor the chemical impacts on organisms, several transgenic animals, such as medaka and zebrafish, expressing a reporter gene have been developed, which can be used as chemical monitors directly in the environment (Kurauchi et al., 2005; Zeng et al., 2005). However, application of these technologies on crustaceans is still very limited. *Daphnia*, being the first species among crustaceans (Kato et al., 2010, 2012) whose genome sequence has been revealed (Colbourne et al., 2011), is a promising model to monitor ecdysteroidal activity with the use of genetic engineering technology.

Ecdysteroidal activity can be estimated by monitoring its action on ecdysteroid receptors. Ecdysteroid receptors are composed of two subunits: ecdysone receptor (EcR), a nuclear receptor that is a ligand-dependent transcription factor, and the nuclear receptor, ultraspiracle (USP). EcR functions by forming a heterodimer with USP (Yao et al., 1993), and the heterodimer binds to a specific target sequence, the ecdysone response element (EcRE). To activate the ecdysone responsive gene, expression of EcR, USP, and an effective concentration of ecdysone is essential. Thus, in order to investigate the active ecdysone signal, the EcRE gene may be placed upstream of a marker gene such as the green fluorescent protein (GFP) gene.

Monitoring of ecdysteroidal activity using live crustaceans is beneficial since it can detect the endogenous ecdysteroidal activity

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during development and maturation, and also evaluate the hormonal activities in the environment. As the molecular functioning of ecdysteroids is not well described in crustaceans, especially in developmental stages, it may also be useful for clarifying the spatio-temporal functioning of ecdysone. In addition, hormonal activity found in the environment is of great concern for environmental protection because the hormonal activities of a chemical compound may be effective at much lower doses than their acute toxicity index (Crisp et al., 1998). Thus, we aimed to introduce a reporter gene into *Daphnia* to monitor its ecdysteroidal activity.

2. Materials and methods

2.1. Daphnia strain and stock culture

The *Daphnia magna* strain used in this study was obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan) (Tatarazako et al., 2003). Cultures of 120 individuals in 5 L medium were maintained at 23 ± 1 °C under a 16-h light/8-h dark photoperiod. Artificial *Daphnia* medium (ADaM) was prepared using reverse osmosis (RO) water and was used as a medium (Klüttgen et al., 1994). Daily culture feedings consisted of a 150-μL suspension of 1.5×10^{10} cells/mL *Chlorella vulgaris* and a 150-μL suspension of 0.15 g/mL yeast. The juveniles were removed every day.

2.2. Plasmids

To generate the 4xEcRE-H2B-GFP plasmid, a nucleotide sequence encoding the luciferase gene of the plasmid 4xEcRE-TATA-Luc (Sawatsubashi et al., 2004), which has four tandemly repeated ecdysone response elements with a minimal promoter, was introduced upstream of the *H2B-GFP* fusion gene (Kato et al., 2012) by using the In-fusion HD Cloning Kit (Clontech Laboratories, Inc., CA, USA).

2.3. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Embryos were obtained from two-week-old *D. magna*. The time when the embryos were laid into the brood chamber (oviposition) was assigned as 0 h. After the collection of the embryos at 0 h, 6 h, 9 h, 12 h, 15 h, 18 h, and 24 h, RNAs were purified and quantitative PCR was performed as described previously (Kato et al., 2008). The copy number was calculated based on the number of embryos instead of using L8 expression for normalization unless otherwise mentioned. Primer sequences are shown in Table 1.

2.4. Microinjection

Microinjection was carried out as described previously (Kato et al., 2011). The plasmid DNA (62.5 ng/μL) was injected into the embryos and the embryos of different stages were incubated in a 96-well plate. For co-injection, the plasmid DNA (62.5 ng/μL) and 1.1 mM of 20-hydroxyecdysone (20E) (≥95%, Cosmo Bio Co. Ltd, Tokyo, Japan) or 1.1 mM of cucurbitacin B (≥98%, Sigma–Aldrich Japan, Tokyo, Japan) was injected into embryos and the embryos were incubated in the medium. To detect endogenous ecdysteroid activity, ten eggs were injected with only DNA and nine eggs were injected with DNA and cucurbitacin B. Ten eggs were injected with DNA and 20E to estimate exogenous ecdysteroid activity. GFP expression was detected under a Leica MZFLIII fluorescence stereoscopic microscope (Leica Microsystems; Heidelberg GmbH; Mannheim, Germany) equipped with a 480-nm excitation filter, a 510-nm barrier filter (filter set GFP2), a 545-nm excitation filter, and a 620-nm barrier filter (filter set DSR). Fluorescent images were recorded with a color digital camera (Leica DC500) mounted on the microscope.

Table 1
Primer sequences for quantitative real-time PCR.

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
EcR-A	CCATGTCGATGAGGGCAGA	CCGAAGGCGGTAAGGTAGAA
EcR-B	CACCACAACCAACTGCATTTAC	CCATTAATGTCAAGATCCCACA
HR3	AGCCGGACGTGTCTCTTCA	ACCTTTGGGCGCATTCTAAC
L32	GACCAAAGGGTATTGACAACAGA	CCAACTTTTGGCATAAGGTACTG

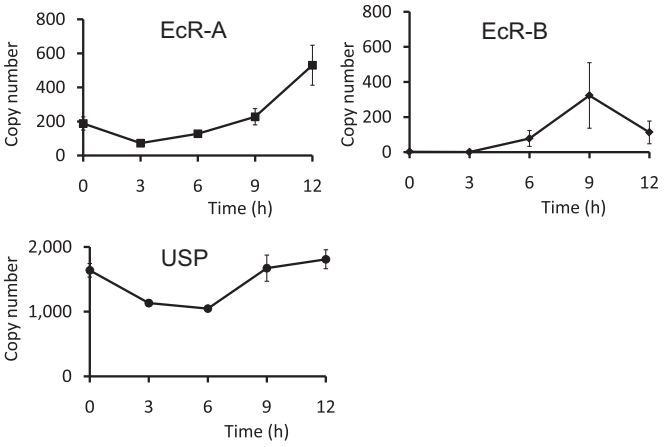

Fig. 1. Temporal expression pattern of *EcRs* and *USP* in *Daphnia* embryos. Expression levels were examined during embryogenesis. Ovulation was assigned as 0 h. Gene expression levels at each time point were converted to copy numbers using calibration curves, and the values were divided by the number of embryos examined. Bars indicate S.D.s.

2.5. Quantification of GFP signal

To quantity the GFP signal, fluorescence intensities were calculated from the images using ImageJ software. For quantification, we used the following formulas:

Total embryo fluorescence = sum of the intensities of all pixels of one embryo;
Background fluorescence = mean intensities per pixel for a region close to the embryo;
Corrected Total Embryo Fluorescence (CTEF) = total embryo fluorescence − (number of pixels of the selected embryo × mean of three background fluorescence measurements);
Relative fluorescence intensity (RFI) = CTEF (injected embryo)/CTEF (uninjected embryo of the same age and of the same mother as the injected embryo).

The image parameters were determined to 90× magnification, 100% aperture, 1.0 s exposure, 1.5 gain, 1.5 saturation, and 0.7 gamma.

3. Results and discussion

3.1. Embryonic expression of the ecdysone receptor

In order to examine if *Daphnia* embryos can be used to monitor ecdysone activity, we examined if EcR was expressed in the embryos. EcR mRNA expression levels at embryonic stages were