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Genetic Engineering for Detection of Endocrine Disruption using I-18 C Gene Expression in *Chironomus riparius*

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Abstract - The 2D/E gel analysis for polypeptide expression reflecting I-18 C gene (early-ecdysterone inducible gene) has conducted the emerged *C. riparius* adults from larval phase exposure to tebufenozide acting as an ecdysteroidal molting hormone. Control group, the amount of ORF II of the I-18 C gene was larger than that of ORF I of this gene. After treatments, ORF I of the I-18 C gene was overexpressed as the polypeptide, whereas ORF II of this gene was expressed as the polypeptide and was clearly reduced expression. Accordingly, we consider that tebufenozide exhibited endocrine disruptions related processing of ecdysteroid receptor protein reflecting ORF II of I-18 C gene. Also, earlier emergence day was related overexpressed polypeptide reflecting ORF I of I-18 C gene. In this study result, tebufenozide induced changing of physiological condition, and then polypeptide expression reflecting early-ecdysterone inducible I-18 C gene was different between control group and exposure group.

Key words : *Chironomus riparius*, I-18C gene, polypeptide expression, tebufenozide

INTRODUCTION

The I-18 C gene belongs to the early-ecdysterone inducible genes that have the regulatory character (Lezzi *et al.* 1989). The I-18 C region of the polytene chromosome of *Chironomus tentans* is a part of the insect chromosome where the Balbiani ring formation occurs and where the I-18 C gene is located (Hertner *et al.* 1986; Lezzi *et al.* 1989). All the transcripts of the I-18 C gene have the same transcription start point and thus have a common promoter. The promoter of the I-18 C gene contains the ecdysterone regulatory elements and also the heat-shock regulatory elements. The I-18 C gene is a model for the study of the eukaryotic gene expression (Lezzi *et al.* 1989, 1991). This gene is activated by heat-shock and the steroid hormone-ecdysterone (Hertner *et al.* 1986; Lezzi *et al.* 1989; Dorsch-Häsler *et al.* 1990). It should be also noted that the very essential information, regarding the regulation of insect genes by ectysteroids, is provided by research on the ecdysone receptor (EcR) that has been conducted (Turberg and Spindler 1992; Wegmann *et al.* 1995; Vögtli *et al.* 1998, 1999; Elke *et al.* 1999; Mouillet *et al.* 2001; Lezzi *et al.* 2002).

Non-steroidal ecdysone agonists have been found in certain plant protection studies that interact with the ecdysteroid receptor complexes of the target species. The action of the agonists, based on the known examples of these compounds, causes the cessation of feeding of the intoxicated larvae within 3~12 hours and the induction of moult that is lethal. The insecticide tebufenozide (N-tert-butyl-N′-[4-ethyl-benzoyl]-3, 5-dimenthyl-benzohydrazide, formerly RH-5992), belongs to the group known as the insect growth regulators, and the benzoylhydrazines have been extensively studied. The benzoylhydrazines have been reported to function at the molecular level as agonists of ecdysteroidal molting hormones, and to exert a variety of

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hormonal effects in insects and crustacean arthropods (Wing 1988; Clare *et al.* 1992; Retnakaran *et al.* 1995; Dhadialla *et al.* 1998). In addition, tebufenozide is a non-steroidal agonist of 20E (20-hydroxyecdysone; molting hormone) and exhibits their insecticidal activity via interactions with the ecdysteroid receptor proteins.

Chironomus riparius (Chironomidae), is a test species which has been extensively used in environmental assessment schemes and standardized chronic assays (USEPA 1994), and has a well studied endocrine system. In this study, we used *C. riparius* which were selectively exposed to a non-steroidal agonist of 20 E, tebufenozide. This study focused on tebufenozide acting as an ecdysteroidal molting hormone and evaluated changing protein expression through hormonal effects on the target insect, *C. riparius*. This study aims to detect changing of polypeptide expression of DNA fragments reflecting open reading frames (ORF) I and II in *Chironomus riparius* exposure to a endocrine disruption chemical, tebufenozide.

MATERIALS AND METHODS

Conditions were consistent with the suggestions for a standard procedure submitted by Streloke and Kopp (1995). *C. riparius* egg masses were reared in an environmental chamber, under long-day conditions with a light : dark cycle of 16 : 8 hours, and a light intensity of about 500 lx. Water temperature was maintained at 20 ± 1°C in an incubation chamber (Sanyo MIR-553, Japan). Twenty fourth-instar larvae were introduced into each test vessel. In order to perform the toxicity test, animals were kept in 300 mL crystallizing dishes (Schott Duran, Germany) filled with 200 mL of M 4 (Elendt and Bias 1990), and a 1 cm sediment layer of fine sand (< 63 μm particle size). The test vessels were aerated continuously after midge larvae were introduced. Water loss due to evaporation was negligible, but when necessary, vessels were refilled with new M 4. Each vessel was provided with 10 mg of ground fish food (Tetra-Werke, Melle, Germany). To achieve an exposure to constant substance concentrations throughout the midges' pupal phase, and to ensure that excess food did not alter water quality, M 4 was removed daily and replaced by new M 4. To prevent the escape of adults during test periods, each vessel was covered with a 0.5 mm mesh net.

Tebufenozide (Sigma-Aldrich Laborchemikalien GmbH, 99.9%) was dissolved in analytical-grade acetone in order to create a stock concentration of 20 mg L^{-1} active ingredient. The nominal concentrations were the control, and 10 μg L^{-1}. *C. riparius* adults were homogenated directly by a motor-driven homogenizer (PowerGen125, Fisher Scientific), and then the protein pellet was solubilized in sample buffer composed of 7 M urea, 2 M thiourea containing 4% (w/v) 3-[(3-cholamidopropy) dimethy-ammonio] -1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte and 1 mM benzamidine. Proteins were extracted for one hour at room temperature with vortexing. After centrifugation at 15,000 g for one hour at 15°C, insoluble material was discarded and the soluble fraction was used for two-dimensional gel electrophoresis. Protein loading was normalized by Bradford assays (1976). IPG dry strips were equilibrated for 12 ~ 16 hours with 7 M urea, 2 M thiourea containing 2% 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% pharmalyte, and then respectively loaded with 200 μg of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences), according to manufacturer's instructions. For IEF, the voltage was increased in a linear fashion from 150 to 3,500 V for 3 hours for the sample entry, followed by a constant 3,500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and then with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 ~ 24 cm, 10 ~ 16%). SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences) in accordance with the manufacturer's instructions. 2D gels were run at 20°C for 1.7 kVh. The 2 D gels were then silver-stained as described by Oakley *et al.* (1980) but the fixing and sensitization step with glutaraldehyde was omitted. Quantitative analysis of digitized images was carried out using PDQuest software (version 7.0, BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots in which the expression level was 2-fold or more than the control value were selected. Each protein was then compiled according to pI and molecular weight (MW).

RESULTS AND DISCUSSION

Organization of the I-18 C gene and the DNA fragments reflecting ORFs I and II

The I-18 C gene of *C. tentans* is composed of three exons and intron sequences with about 7.9 kbp length. This gene produces at least two main transcripts (1.8 and 4.6 kb RNAs). ORF I is located in 1.8 kb RNA and its sequence reflects a part of intron 1 of that gene, whereas ORF II is contained in 4.6 kb RNA and its sequence reflects a part of exon 3 of the I-18 C gene. ORF I is 420 bp long and ORF II is 273 bp long. The transcription of the I-18 C gene can be stimulated by ecdysterone (Hertner *et al.* 1986), whereas

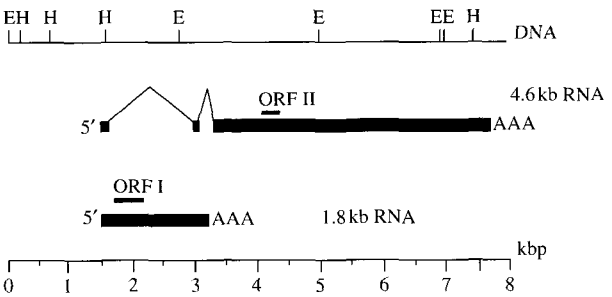

Fig. 1. The organization of the I-18 C gene (based on Amrein *et al.* 1988; Lezzi *et al.* 1989; Dorsch-Häsler *et al.* 1990; Borowicz 2005). The top line shows the restriction map of the genomic DNA with the indication of the restriction site of the Eco RI (E) and Hind III (H). Two main transcripts of this gene are shown ORF I present in 1.8 kb RNA and ORF II present in 4.6 kb RNA.

1720
ATG GTT GCT ACG TCT TTG CAT AGT AAT GGA AAT AAA ATA ATT CAC
met val ala thr ser leu his ser asn gly asn lys ile ile his

ACA GAG GCT TTA AAT GAG TGG ATA AGA AAT TTA AAA TCA AAG AAA
thr glu ala leu asn glu trp ile arg asn leu lys ser lys lys

CAA TTT AAG AAA AAG ATC TCG CGA GGC CAA GAC CTC TTT GCA ATA
gln phe lys lys lys ile ser arg gly gln asp leu phe ala ile

GCC ATT TTA CAA AAT TCA TTA TAT AAA AAT CAG AAA ATC CTT CAA
ala ile leu gln asn ser leu tyr lys asn gln lys ile leu gln

CAA AGA CAA AGA GAG AGA TCT GAG AAA TGG CTT AAA ATG AAG AAA
gln arg gln arg glu arg ser glu lys trp leu lys met lys lys

ATG CTT GAA GAA AGC ACT AAA AAT TGT GAT AAT TCT GAG TAC ATG
met leu glu glu ser thr lys asn cys asp asn ser glu tyr met

TCC TCA GAG GAT GAT AAA GAC TAC CAT CTT GAG AGT CTA GAA CGT
ser ser glu asp asp lys asp tyr his leu glu ser leu glu arg

GAA GAG AGA CAG ATG CAG ATT AAG AAA ATC TGG GAA GAA GAT TTA
glu glu arg gln met gln ile lys lys ile trp glu glu asp leu

AGT GAT ATT GAG AGC TTT ATG AAT AGT TTA GAT ACA GTT AAA ACA
ser asp ile glu ser phe met asn ser leu asp thr val lys thr

2139
TCA TTA GTT AGA TAA
ser leu val arg OCH

Nde I
The 5′ end (F) primer: GCG CAT ATG GTT GCT ACG TCT TTG CAT AG...
The sequence of DNA fragment reflecting ORF I

Bam HI
The 3′ end (R) primer: GCG GGA TCC TCT AAC TAA TGA TGT TTT AAC...
The sequence of DNA fragment reflecting ORF I

Fig. 2. The sequence of DNA fragment reflecting open reading frame I of the I-18 C gene (Amrein *et al.* 1986) and the design of primers for its isolation using the PCR.

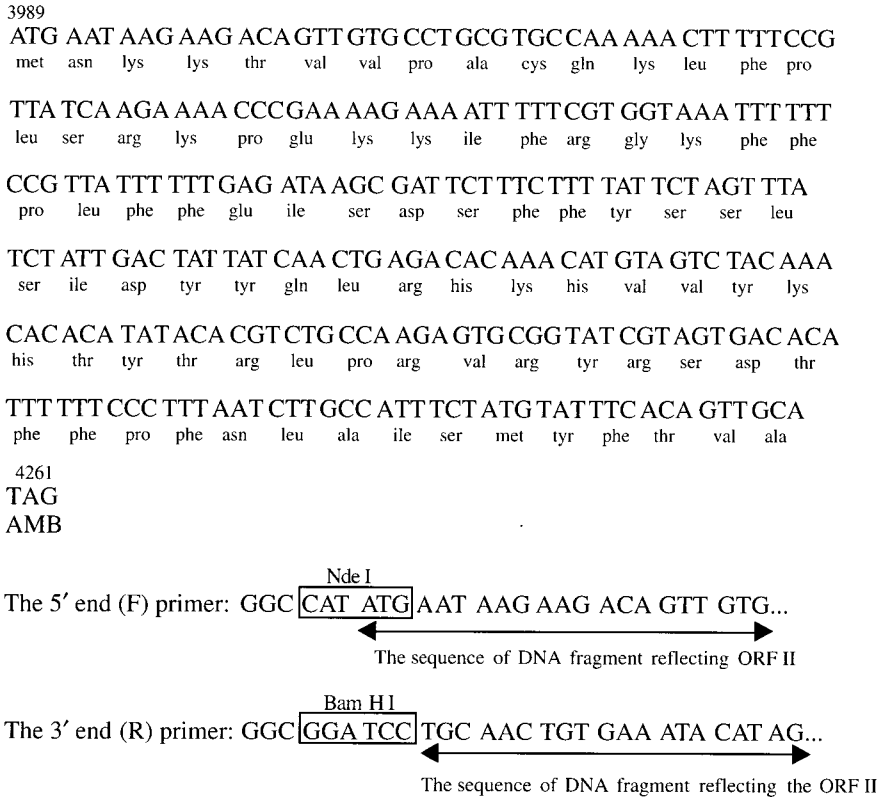

Fig. 3. The sequence of DNA fragment reflecting open reading frame II of the I-18 C gene (Amrein *et al.* 1986) and the design of primers for its isolation using the PCR.

the other transcript of that gene is heat-shock inducible (Dorsch-Häsler *et al.* 1990). The organization of the I-18 C gene of *C. tentans* with indication of the main transcripts and the localization of ORFs I and II of that gene are shown in Fig. 1. The DNA sequence of that gene was determined, as well as its structural and developmental organization (Hertner *et al.* 1986; Amrein *et al.* 1986, 1988; Lezzi *et al.* 1989; Dorsch-Häsler *et al.* 1990).

The sequence of DNA fragments reflecting ORFs I and II of the I-18 C gene is shown in Fig. 2 and 3, respectively. The DNA fragments reflecting ORFs I and II were amplified by the PCR technique using the existing templates (DNA fragments of the I-18 C gene of 2.8 and 2.1 kb length) respectively, that had been cloned into the pUC8 vector, the length of which alone was 2.9 kb (Messing and Vieira 1982; Helfman *et al.* 1983). The pair of oligonucleotide primers used to amplify the DNA fragment reflecting ORF I by the PCR technique, includes parts of intron 1 of the I-18 C gene, whereas the pair of primers, used for the PCR amplification of the DNA fragment reflecting ORF II, includes parts of exon 3 of the I-18 C gene. The primer design was performed based on the published sequence of the I-18 C gene by Amrein *et al.* 1986. Using the PCR technique the whole sequence of the DNA fragments reflecting both ORFs was amplified. The DNA fragments reflecting the particular ORFs of the I-18 C gene, to be obtained by the PCR, should be suitable to the translational system of the T7 RNA polymerase/promoter. These DNA fragments (the inserts) should have the Nde I and Bam HI restriction sites (at their 5′ and 3′ ends, respectively). Thus, these restriction sites were additionally constructed in the oligonucleotide primers (Figs. 2 and 3).

The DNA fragment of ORF I of the I-18 C gene was consisting of 140 codons, should code a polypeptide of 16,616 Daltons and also the DNA fragment reflecting ORF II of the I-18 C gene consisting of 91 codons, should code a polypeptide of 10,925 Daltons when translated. The intact native proteins of ORFs I and II of the 1.8 and 4.6 kb RNA of the I-18 C gene of *C. tentans* was achieved 21.5 and 14.4 kD in electrophoretic analysis (Borowicz 2005).

The polypeptide expression and change of emergence day

In the preliminary study, *C. riparius* were placed into various concentrations (control, 10, 30, 60, 100 μg L^{-1}) of tebufenozide (LC_{50} 81.94 μg L^{-1} and NOEC 30 μg L^{-1}; Hahn *et al.* 2001, Kwak and Lee 2004, 2005). The mortality of control groups was little different from that of the low concentration (10 μg L^{-1}). In the experimental process, the body volume of exposed female was thinner than that of the control female but those of male adults didn't show a difference (Kwak and Lee 2005). Recent, Kwak and Lee (2005) reported that the difference of protein expression in *C. riparius* was induced after treatment of tebufenozide acting as an ecdysteroidal molting hormone using in two-dimensional gel electrophoresis analysis.

Especially this study was focused that polypeptide expression of I-18 C gene (early-ecdysterone inducible gene) was observed adult of *C. riparius* exposure to tebufenozide using 2D/E. Control group, the amount of ORF II of the I-18 C gene was larger than that of ORF I of this gene (Table 1). After treatments, ORF I of the I-18 C gene was overexpressed as the polypeptide, whereas ORF II of this gene was expressed as the polypeptide and was clearly reduced expression (Table 1). Similar results reported that in *C. tentans*, ORF I of the I-18 C gene was overexpressed as the polypeptide, whereas ORF II of this gene was expressed as the polypeptide and was strongly visible (Borowicz 2005).

The amounts of the different transcripts of the I-18 C gene (1.8 and 4.6 kb RNAs) do not change similarly during the development and under the experimental induction. This feature may be related to the complexity of the promoter of this gene and changes in RNA processing that are caused by different physiological conditions, such as heats-hock, etc. (Lezzi *et al.* 1989). In this study result, tebufenozide, an endocrine disruption chemical, induced changing of physiological condition, and then polypeptide expression reflecting early-ecdysterone inducible I-18 C gene was different between control group and exposure group (Table 1).

I-18 C gene, early-ecdysterone inducible gene, should be affect emergence day of *C. riparius*. Emergence day of treated group in male and female *C. riparius* was faster than that of control group (not treated group). Especially, ORF I of I-18 C gene was clearly increased after treatments. Accordingly, we consider that tebufenozide exhibited endocrine disruptions related processing of ecdysteroid receptor protein reflecting ORF II of I-18 C gene. Also, earlier emergence day was related overexpressed polypeptide reflecting ORF I of I-18 C gene. Further study should be conduct to confirm relationship between emergence day and polypeptide expressions reflecting ORF I and ORF II of I-18 C gene. The expressed polypeptides of ORFs I and II of both RNAs, respectively, of the I-18 C gene of *C. riparius* could be used to biomarker of endocrine disruption. The expression of that gene should be explain mechanisms of its expression in general and needed to continue studies regarding the expression of this gene in terms of eco-toxicology.

Table 1. The amount of expressed polypeptide of DNA fragment reflecting open reading frame I (ORF I; 21.5 kD) and open reading frame II (ORF II; 14.4 kD) of the I-18 C gene of *C. riparius* was changed after insecticide treatment

kD	Isoelectric point	Control		Treatments	
		Male	Female	Male	Female
14.4	6.1	692.3	496.8	317.7	0
21.5	6.0	0	224.2	327.2	652.8

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