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cDNA Library Construction in the Multicolored Asian Ladybird Beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae) for Gene Functional Analysis Using Gateway Cloning System

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In this preliminary study, we sought to investigate the genes involved in the differentiation of elytral color and pattern in the beetle *Harmonia axyridis*. We confirmed the inhibition of ecdysis after knocking down *H. axyridis* ecdysone receptor A, and generated an *H. axyridis* cDNA library using the Gateway system for functional analysis of undiscovered genes in *H. axyridis*. We generated an *H. axyridis* cDNA library with a titer of 4.43×10^4 and insert sizes of approximately 200–700 bp. Examination of the genetic information of this cDNA library showed that most sequences were from insect genes and one was an *H. axyridis* gene. After cloning *H. axyridis* ecdysone receptor A into a LITMUS 28i vector, 400–500 bp double-stranded RNA was synthesized using the T7 promoter. When the synthesized double-stranded ecdysone receptor A was injected between the 1st and 2nd sections of the abdomen of 0 to 2-day-old 4th-instar larvae of *H. axyridis*, most of the larvae died within 4 days after injection, whereas others failed to pupate. qRT-PCR analysis showed that, compared with non-treatment and distilled water treatment groups, the expression level of ecdysone receptor A in larvae injected with double-stranded RNA of ecdysone receptor A decreased markedly at day 4 after injection.

Key words: cDNA library, Ecdysone receptor, Gateway cloning system, *Harmonia axyridis*

INTRODUCTION

Harmonia axyridis belongs to the family Coccinellidae in the order Coleoptera. Worldwide, approximately 490 genera and 4,200 species of Coccinellidae have been recorded (Iperti, 1999), among which 74 species have been reported in Korea (ESK, 1994), 400 species in North America (Belicek, 1976), and 110 species in Europe (Iperti, 1986). *H. axyridis* is known to be distributed throughout the world from North America to Europe in addition to Far East Asia (Brown and Miller, 1998; Chapin and Brou, 1991; Day *et al.*, 1994; Kidd *et al.*, 1995; Lamana and Miller, 1998; Nalepa *et al.*, 1996; Tedders and Schaefer, 1994). This beetle is a beneficial insect with high economic value since it can be used for the effective control of aphids, upon which it preys (Lee and Kang, 2004; Tedders and Schaefer, 1994; Watanabe, 1964). In addition to its role as a biological control agent of aphids, *H. axyridis* has attracted interest with regards to determining the factors underlying the diverse colors and patterns on the elytra of this species. In general, the elytra have either a maximum of 19 spots on a yellowish orange background or red spots on a black background (Korschefsky, 1932). Regarding the polymorphism of elytral patterns in *H. axyridis*, Mackauer (1976) suggested that the color and

size vary depending on regional distribution. It has also been reported that the amount of food fed during the larval stage can affect the expression of colors and patterns (Grill and Moore, 1998), and that the temperature and humidity conditions during the pupal stage can affect the elytral color expression of the imago (Sakai *et al.*, 1974). Furthermore, Kang *et al.* (2009) presumed that temperature, photoperiod, and the types of aphid provided as food under indoor conditions do not have strong effects on elytral colors and reported that the elytral color pattern is affected by average outdoor temperature and duration of sunshine during larval development.

Hence, the precise factors causing the differentiation in elytral colors and patterns in *H. axyridis* have yet to be conclusively determined. Therefore, from a molecular perspective, to examine which genes are involved in determining the elytral colors of *H. axyridis*, specific genes were knocked down by RNA interference (RNAi) in this study to inhibit the expression of elytral colors or patterns in *H. axyridis*.

RNAi refers to gene silencing induced by double-stranded RNA (dsRNA). The dsRNA introduced inside the cell is digested by the enzyme dicer to produce 21–25 nucleotides, and the excised siRNA binds to an RNA-induced silencing complex (RISC), which then binds to the complementary mRNA and inhibits the expression of target mRNA (Bellés, 2010; Price and Gatehouse, 2008). The RNA inhibition of gene expression was first discovered in *Caenorhabditis elegans* in the late 1990s (Fire *et al.*, 1998), and RNAi studies have since been conducted in various insects, including those in the orders Blattodea (Martin *et al.*, 1995), Diptera (Attardo *et al.*, 2003; Misquitta and Paterson, 1999),

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Coleoptera (Arakane *et al.*, 2004; Brown *et al.*, 1999; Bucher *et al.*, 2002; Suzuki *et al.*, 2008; Tomoyasu and Denell, 2004), Hemiptera (Jaubert-Possamai *et al.*, 2007), Hymenoptera (Amdam *et al.*, 2003; Lynch and Desplan, 2006), Lepidoptera (Chen *et al.*, 2008; Dai *et al.*, 2008; Hossain *et al.*, 2008; Quan *et al.*, 2002; Rajagopal *et al.*, 2002), and Orthoptera (Dong and Friedrich, 2005; Miyawaki *et al.*, 2004). To deliver dsRNA inside the body of an insect, it can either be injected or administered in the diet. The injection of dsRNA has been studied in various insects, including fruit flies (Kennerdell and Carthew, 1998), red flour beetle (Brown *et al.*, 1999), Gambian mosquito (Blandin *et al.*, 2002), silkworm moth (Ohnishi *et al.*, 2006; Tabunoki *et al.*, 2004), honeybee (Aronstein and Saldivar, 2005; Farooqui *et al.*, 2003; Gatehouse *et al.*, 2004), *Acyrtosiphon pisum* (Jaubert-Possamai *et al.*, 2007), German cockroach (Bellés, 2010; Huang and Lee, 2011; Martin *et al.*, 1995), cricket (Moriyama *et al.*, 2008), and two-spotted spider mite (Grbić *et al.*, 2011; Khila and Grbić, 2007). Feeding of dsRNA has been studied in tortricid moth larvae that have biting mouth parts instead of sucking mouthparts (Turner *et al.*, 2006), *Helicoverpa armigera* (Kumar *et al.*, 2009), *Diabrotica virgifera virgifera* (Bolognesi *et al.*, 2012), and *Plutella xylostella* (Bautista *et al.*, 2009).

Recently, RNAi has been considered not only as an alternative strategy for agricultural pest control but can also be effectively used to study the function of genes. In the present study, in order to determine which genes are involved in the differentiation of elytral colors and patterns in *H. axyridis*, the ecdysone receptor A gene of this beetle was knocked down, which inhibited ecdysis, and a cDNA library of *H. axyridis* was generated using the Gateway system for functional analysis of undiscovered genes in *H. axyridis*.

MATERIALS AND METHODS

Insects

An overwintering population of *H. axyridis* collected from Chungnam Geumsan-gun in 2014 was placed in a 15 × 20 cm plastic petri dish and maintained in a 15°C incubator with provision of artificial food.

cDNA library construction

A cDNA library was generated using the SuperScript Full length cDNA library Construction Kit II protocol (Invitrogen, USA) with modifications.

Total RNA extraction and mRNA isolation

Total RNA of *H. axyridis* was extracted using Trizol reagent (MRC, USA) and was dissolved in 30 µl of diethyl pyrocarbonate (DEPC)-treated water as the last step. mRNA was separated from the total RNA using a FastTrack® MAG mRNA Isolation Kit (Ambion, USA) and was stored at -80°C.

cDNA preparation

The primers used to synthesize the first-strand cDNA were generated by adding sites for the restriction enzymes *EcoRI* and *HindIII* to the primers indicated in the SuperScript® Full Length cDNA Library Construction Kit (Table 1). To 25.5 µl of mRNA, we added 2 µl of the First-strand synthesis primers, and the mixture was heated at 70°C for 7 min and then incubated at room temperature for 10 min. During this incubation period, 10 µl of 5× First-strand buffer, 5 µl of 0.1 M DTT, 2.5 µl of 10 mM (each) dNTPs, and 5 µl of SuperScript® III RT (200 U/µl) were added to a 0.2-ml PCR tube to generate a first-strand mixture, which was incubated at 45°C for 2 min. The priming mixture and the first-strand mixture were then mixed together, and first-strand cDNA was synthesized by heating the reaction mixture at 45°C for 20 min, 50°C for 30 min, and 55°C for 30 min. Residual mRNA was removed by RNase I (Ambion, USA) treat-

Table 1. A list of the primers used in cDNA library construction

Clones	5'-Oligo	5'-Oligo sequence	3'-Oligo	3'-Oligo sequence
First strand synthesis			Biotin-attB2-(N) ₂₅	Biotin-GGG GAC AAC TTT GTA CAA GAA AGT TGG GAA GCT T(N) ₂₂
AttB1_adapter ligation (double-strand)	attB1-adapter-UP	TCG TCG GGG ACA ACT TTG TAC AAA AAA GTT GGG AAT TC		
	attB1-adapter-DOWN	PGAA TTC CCA ACT TTT TTG TAC AAA GTT GTC CCC		
Second strand synthesis		TCG TCG GGG ACA ACT TTG TAC AAA AAA GTT GGG AAT TC		
attB-flanked DNA PCR		Biotin-GGG GAC AAC TTT GTA CAA GAA AGT TGG G(N) ₂₂ AAG CTT VN		TCG TCG GGG ACA ACT TTG TAC AAA AAA GTT GGG AAT TC
	pDONR 207 F	TCG CGT TAA CGC TAG CAT GGA TCT C	pDONR 207 R	GTA ACA TCA GAG ATT TTG AGA CAC

ment.

An attB1 adapter was attached to the 5' end of the first-strand cDNA (Table 1). Thereafter, 22 μ l of cDNA, 10 μ l of 5 \times Adapter Buffer, 5 μ l of 5' Adapter Mix (0.5 μ g/ μ l), 8 μ l of 0.1M DTT, and 5 μ l of T4 DNA Ligase (1 U/ μ l) were mixed together to give a total volume of 50 μ l, which was then reacted at 16°C for 20 h.

To 79 μ l of first-strand cDNA with attached attB1 adapter, we added 10 μ l of 10 \times High Fidelity PCR buffer, 4 μ l of 10 mM (each) dNTPs, 5 μ l of 50 mM MgSO₄, 1 μ l of Second-strand synthesis Primer (100 ng/ μ l) (Table 1), 1 μ l of High Fidelity Platinum® Taq DNA Polymerase, and 5 μ l of distilled water (total volume 100 μ l), which was reacted at 68°C for 20 min and 72°C for 40 min to generate a second strand.

PCR

To amplify cDNA, attB-flanked DNA PCR primers were generated using the attB1 and attB2 sequences (Table 1). The cDNA of *H. axyridis* was amplified using the generated primers and LA Taq® DNA Polymerase (Takara, Japan). The reaction conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, which was repeated for 20, 25, or 30 cycles and ended with the extension at 72°C for 5 min.

BP recombination reaction

BP recombination was conducted between the cDNA and a pDONR 207 vector. After 20 h of reaction at 25°C, 2 μ l of Proteinase K was added and reacted at 37°C for 15 min and then 75°C for 10 min to terminate the BP recombination reaction.

The sample that underwent BP recombination was transformed into One shot® Top10 Electrocomp™ *Escherichia coli* (Invitrogen, USA) using an electroporator. The electroporation conditions were as follows: voltage, 1.8 kV; resistance, 200 Ω ; and capacity, 25 μ F. The *E. coli* cells were incubated in S.O.C media for 1 h. The resulting transformants were serially diluted from 10⁻¹ to 10⁻⁴ and 100- μ l aliquots were plated onto gentamycin-containing media. After overnight incubation at 37°C, the cDNA library titer was confirmed from the number of colonies formed. The cDNA library material remaining after plating was aliquoted at 300 μ l, mixed with the same volume of 40% glycerol, and stored at -80°C.

RNAi of *H. axyridis* ecdysone receptor A

Ecdysone receptor A preparation

Total RNA was extracted from an imago of *H. axyridis* using Trizol reagent (MRC, USA) and dissolved in 20 μ l of DEPC-treated water. Using the extracted total RNA as a template, cDNA was generated using ReverTra Ace- α -® kit (TOYOBO, Japan).

The cDNA of *H. axyridis* ecdysone receptor A was amplified using Prime Taq DNA polymerase (GENETBIO, Korea). The primers used for this reaction were generated with reference to GenBank accession number AB506666.1, and sequences for digestion with the

restriction enzymes *Eco*RI and *Hind*III were added to the primers to facilitate cloning of the cDNA in a LITMUS 28i Vector (NEB, USA) (Table 1). Amplification was performed in a 20- μ l reaction mixture, containing 3 μ l of cDNA, 2 μ l of 10 \times Reaction Buffer, 2 μ l of 10 mM dNTP mixture, 1 μ l of HaEcRA FW and HaEcRA RV primers, 0.2 μ l of Prime Taq DNA polymerase, and 10.8 μ l of distilled water in a 0.2-ml PCR tube. The reaction conditions were as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, which was repeated for 30 cycles and ended with extension at 72°C for 5 min.

Ecdysone receptor A::T-Blunt vector cloning

The ecdysone receptor A PCR product was cloned into a T-Blunt vector using a T-Blunt™ PCR Cloning Kit (SolGent, Korea). Four microliters of PCR product, 1 μ l of 6 \times T-Blunt buffer, and 1 μ l of T-Blunt vector were added to 1.5-ml Eppendorf tubes and the 6 μ l total reaction volume was maintained at room temperature overnight.

One microliter of cloning sample was used to transform *E. coli* (DH5 α) competent cells. The cells were incubated for 1 h and 300- μ l aliquots of the resulting transformants were subsequently plated on Luria-Bertani (LB) plates containing kanamycin (1 μ l/ml), which were incubated at 37°C overnight. Single colonies from the kanamycin plates were incubated in 3 ml of LB broth at 37°C and 250 rpm overnight. Plasmid DNA was subsequently extracted from the cultured cells using a Plasmid Purification Mini Kit (NucleoGen, Korea).

Successful cloning in the T-Blunt vector was confirmed by digestion with *Eco*RI and *Hind*III. Reaction mixtures (10 μ l) containing 8 μ l of plasmid DNA, 0.5 μ l of *Eco*RI, 0.5 μ l of *Hind*III, and 1 μ l of 2.1 buffer were incubated at 37°C for 3 h.

Ecdysone receptor A::LITMUS 28i vector cloning

In order to obtain high-density DNA, prior to cloning *H. axyridis* ecdysone receptor A into the LITMUS 28i vector, both were subjected to electrophoresis and gel extraction using an AccuPrep® PCR & Gel Purification Kit (Bioneer, Korea). *H. axyridis* ecdysone receptor A (25 μ l) was then cloned into the LITMUS 28i vector using a 10- μ l reaction mixture, containing 2 μ l of LITMUS 28i vector, 6 μ l of ecdysone receptor A DNA, 1 μ l of 10 \times Ligase Buffer, and 1 μ l of T4 DNA Ligase (Promega, USA), which was incubated at room temperature overnight.

The cloned sample was used to transform *E. coli* (DH5 α) competent cells. The cells were incubated for 1 h and 300- μ l aliquots of the resulting transformants were plated onto LB plates containing ampicillin (1 μ l/ml) and incubated overnight. Single colonies grown on the ampicillin plates was incubated in 3 mL of LB broth at 37°C and 250 rpm overnight. Plasmid DNAs were subsequently extracted from the cultured cells using a Plasmid Purification Mini Kit (NucleoGen, Korea).

Successful cloning was confirmed by digestion with *Eco*RI and *Hind*III. Reaction mixtures (10 μ l) containing 8 μ l of plasmid DNA, 0.5 μ l of *Eco*RI, 0.5 μ l of *Hind*III,

and 1 μ l of 2.1 buffer were incubated at 37°C for 3 h.

Double-stranded ecdysone receptor A synthesis

The plasmid DNA templates comprising *H. axyridis* ecdysone receptor A cloned in the LITMUS 28i vector were digested with *Hpa*I for linearization. Reaction mixtures (10 μ l) containing 8 μ l of plasmid DNA, 1 μ l of *Hpa*I (Takara, Japan), and 1 μ l of 10×K Buffer were incubated at 37°C overnight.

To use the linear DNA as a template, double-stranded ecdysone receptor A (dsEcRA) was synthesized using a T7 RiboMax™ Express RNAi system kit (Promega, USA). After synthesis, 1 μ l of 200-fold diluted RNaseA and 1 μ l of undiluted DNase were added and reacted at 37°C to remove residual DNA templates and ssRNA. After the reaction, the dsEcRA was concentrated and dissolved in 20 μ l DEPC-treated water.

Double-stranded ecdysone receptor A injection

Thirty to forty 4th-instar *H. axyridis* larvae were allocated to each of three experimental groups: non-treatment group, distilled water treatment group, and dsEcRA treatment group. Before injection of dsEcRA, the larvae were starved for 1 h for efficient absorption of dsEcRA into the body. dsEcRA or distilled water was injected between the 1st and 2nd sections of abdomen of the 4th-instar larvae. Larvae in the distilled water group were injected with 3 μ l of distilled water, whereas those in the dsEcRA group were injected with 1 μ l of dsEcRA. Following injection, the larvae were maintained on a diet of green peach aphid and cotton aphid.

Expression analysis of ecdysone receptor A

Real-time PCR was used to analyze the expression level of the ecdysone receptor A following injection of the 4th-instar larvae with dsEcRA. On days 2, 3, and 4 after injection, three of the 4th-instar larvae were selected from each treatment group, and total RNA was extracted using Trizol reagent. First-strand cDNA was synthesized from 1 μ g of total RNA using ReverTra Ace- α -® (TOYOBO, Japan). A CFX96™ Real-Time System and CFX Manager software (Bio-Rad, USA) were used for real-time PCR. PCR was performed in 20- μ l reaction mixtures containing 2 μ l of 1/5-fold diluted cDNA, 10 μ l of iQ™ SYBR® Green supermix, 0.5 μ l of forward primer, 0.5 μ l of reverse primer (Table 1), and 7 μ l of distilled water. The reactions were carried out under the following conditions: 95°C for 3 min, 95°C for 10 s, 58°C for 10 s, and 72°C for 20 s, which were repeated for 39 cycles, and then reacted at 95°C for 10 s, 65°C for 5 s, and 95°C for 0.5 s. Ribosomal protein 49 (rp49) was used as a housekeeping gene.

RESULTS

Generation of a cDNA library

PCR was conducted with 20, 25, or 30 cycles to determine whether *H. axyridis* cDNA of different sizes could be synthesized. We found that 30 cycles of PCR synthesized cDNAs with more diverse sizes than either 20 or 25 cycles of PCR (Fig. 1).

The number of colonies that grew on gentamycin plates were counted to measure the titer of the cDNA library. Two colonies were found on the 10⁻³ plate, 38 colonies on the 10⁻² plate, and 527 colonies on the 10⁻¹ plate. Finally, an *H. axyridis* cDNA library with a titer of 4.43×10^4 was obtained (Table 2).

To confirm the size of genes in the *H. axyridis* cDNA library, 24 random colonies were selected from the 10⁻¹ gentamycin plate and treated with restriction enzymes. This produced inserts with sizes ranging from 200 bp to 700 bp (Fig. 2). To obtain the nucleotide sequence and genetic information for the 24 samples with validated inserts, sequencing (Macrogen) was requested and an NCBI BLAST search was performed. The results showed that sequences of 18 of the 24 samples were from insect genes, five were from genes of species other than insects, and the final sample was confirmed as a sequence from an *H. axyridis* gene (Table 3).

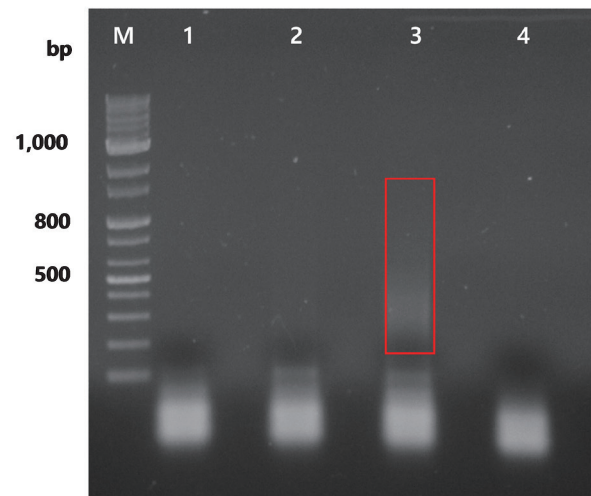


Fig. 1. Electrophoresis of *Harmonia axyridis* cDNA amplified using an N₂₂ random primer and a 5' primer. M: 1-kb DNA ladder marker; Lane 1: PCR product from 20 cycles; Lane 2: PCR product from 25 cycles; Lane 3: PCR product from 30 cycles; Lane 4: Negative control.

Table 2. Final titer of the *Harmonia axyridis* cDNA library

Dilution	Amount Plated (μ l)	Colonies per Plate	Titer* (cfu/ml)	Average Titer* (cfu/ml)	Total Volume (ml)	Total CFUs (cfu)
10 ⁻³	100	2	2×10^5			
10 ⁻²	100	38	3.8×10^5	3.69×10^5	12	3.07×10^4
10 ⁻¹	100	527	5.27×10^5			

* The cDNA library titer was determined by counting colonies

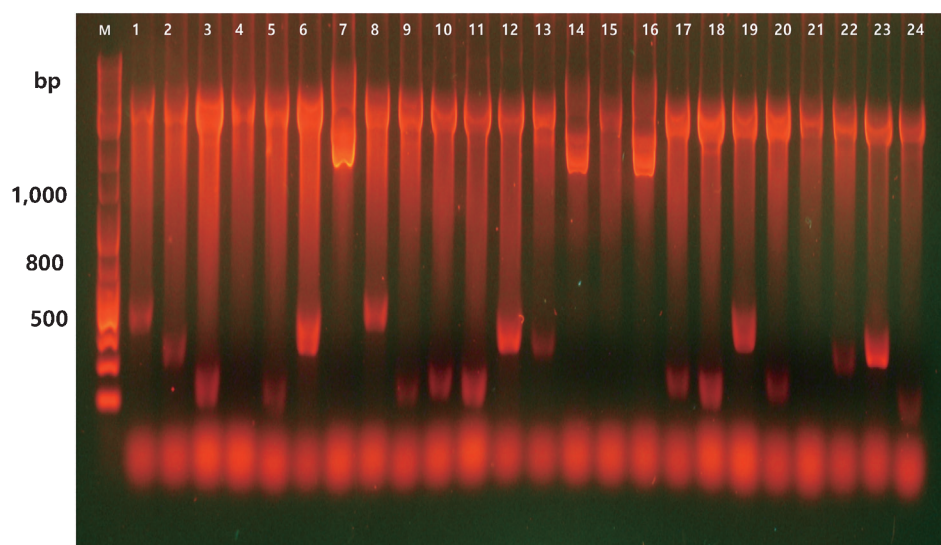


Fig. 2. Electrophoresis of cDNA library inserts. Plasmids from 24 randomly selected colonies were analyzed on agarose gel to determine insert size. The cDNA size was confirmed to be in the range 100 bp–500 bp. M: 1-kb ladder; Lane 1–24: cDNA library inserts.

Table 3. Results of sequencing analysis of cDNA from 24 colonies identified from an NCBI BLAST search

	EST blast search	Sequence	Size (bp)	Identity (%)
No. 1	<i>Asobara tabida</i> hch gene for ferritin heavy chain and hch gene for ferritin light chain	AAGCTTGTGGAACCTTCCCTGTTCATGTCGTAGTTGGCGAAATGAGTGGACATGAGTAAGTATTCAAAGTTCTTGATGATGTGGGTTAGGGCGAATTTATTCAAGTCATCTCGAGGTGACCAATGGCTCCATAGCGGGAGTTACAAGAAGATTCGGTGTGGTG GTGCTTTGTTTATTGATATTTGGTCTGCAAGCATCTTGGTAGCCGTGAGACATTCTTGAGAATTC	232	78
No. 2	<i>Salmo salar</i> clone Contig 4326 Cystatin-B putative mRNA, complete cds	AAGCTTAACTTTGCCCCTGGAGTCGACCACCGCAAATGTTTCTAAGTCAATTGATTTTACATCTTTGTATCATGTTGTATATTGTATTGTACTTAAAAACCCCTTTGTAT TGACGCGACTTTTGATGTAAACGCCACCGTGCAGACAAGAACC GAATAACTTGAGGAACTCAGACCGCATGTGCTCGGGGACAACCTTTGTACAAAAAAGTTGGGAATTC	221	100
No. 3	<i>Sesamum indicum</i> putative late blight resistance protein homolog R1A-10, mRNA	AAGCTTTCCATACTTCTAAAGGTCCAAAACAAGCTTTGCGACAACGGAGATGGTT AGGGGGGGTCCGAAAAGTGTCAATTTCCAAAATCACCTGTGTCTCTTGAACGGA AGCAGTTGTGCAAAATCTGATTGGACCAACTTGAGTTTCACGAAAAAGTAGGACA GGAGCGTGAAAGCCGCAAGGCTCTATCTTAGGTAACAGGCGAGAAACCTGGCTG TCTCACCCAAAATGGGATGCGAATTGGAATTC	251	93
No. 4	<i>Tribolium castaneum</i> trithorax group protein osa, mRNA	AAGCTTTTCGATAGCATCGTCCTGGGTGTCAGGTCTGCCGCCAGTCCGCAGCATCA GGGTTTTCCGAGTAGGCCCCGCCAGCCTACCACACCTAATGCCCATGGACCGGAC GCAGCTGATCTGAGTGGGGGCAATAGCAACGACAGTTCCGGTGGCCCCGATCCCG GGAACGCCTAACTCGCAAGGTATGAGGCCGACCCCTCTCCAACAGGTTCAACGG GGTCCAGGTGATGTCGCCGGCAGTTGGTCAACAGAACATTTCCATGCCACCTCG ACCGTCCAGCGGGCCATCAGACCCTCCTACCAGAATCAGCCACTTCCCATTTGGT TCTGCGCCAGGCAGCTACCCAAGTACACACCCTGCACCGCATCCCTCCCATGCGC CTCACGGGTACAAAACGCATCCAGGGATGACACCATCGGCTCCTCCACCGCGCA ACAGATGACTATATATCAGACCAGCCAACAGTAACTTTGAGAATTC	486	76
No. 5	<i>Tribolium castaneum</i> nuclear transcription factor Y subunit B-4, transcript variant X3, mRNA	AAGCTTTTTTCGATAAGTAGATCACGGCAGAAAATACAGATGATTGCAATCACGA CGGCGGTGATAAACGTGGAGCACCTTTGAGAGAACAAGATAGATTTCTGCCAATT GCAAATGTAGCAAAAATTATGAAGCGTGCCATTCTTGACACAGGAAAAGGTAAATA GCAAAATAGCAAAAGATGCTAGGGGGTGTGTTCAAGAATGCGTTTCAGAGTTTAT CAGTTTTATAACTAGTGAAGCAAGTGATCGTTGTCATATGAAAAAAGGAAGACC ATAAACGGAGAGAACCATGAGAATTC	221	75

No. 6	<i>Tribolium castaneum</i> hypothetical protein TcasGA2_TC014392	AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA TCAAAATAAAATTAAGATAACACATTAGACATTAATCTAAGCTCTACCCCTAAACTG CTTGTGTAATCTGGAAGTGAATCATCATACTGGGCTTGGAAAAAGTTACCAGCT ACTGCATTCCCCAAATTTACTTTTCAGCGAACTTTTTCACCGAAAAATTACCCCT ATCCCATCGGTCTTGGAGTGCTTCGTTTCTTGAAGTCGACCTTACCAGGCTGTT TGAACAGCAGAAAGATATACCTGTGAAGACCGGTATCTTAGGTGGCCCTGATCC CACGTACTCGTTCAATACTTCCCCTTTGGATAAATCAGAACGGGAATATTCAACCAC CAGCCAATGTTGGAATTCGGGGACAACCTTTGTTACGAAGTTATATTCAATTGGTC AGACAAAATTGATTGACCAAAACCCCCATAACAGCAAAATTTCTGCCGTGAG TATGAGCGCGAAAGCATTATAGGTGCGTTTCGGAGCGAGTTAGACGGTCTTACTA ACTCAGTTCCCCCTTCTCTCATTTCGTGTAATCTTCTGTGTATTGTGTATGTGTCT TTGGGGGTAGGCAAAATTTGTGTTAGTTGTAAGGTGTTCTGATACCGATATAGC GATTATTATGTTAGGACATATGCATAATTTGAAAGACGATGGTTCCAATGTATGGT TAAACGCAGGCACCGGAAGCAACCAATATATTAATTTGACAAAAGTGTACGAGA ACTTGGGATCATCTTTGTGTGCAAGTTTCCAGGATTTTCATGCATTAACCCGGAT GTGACTATAATCTCGCTTTTGAATTC	859	77
No. 7	<i>Dendroctonus ponderosae</i> , whole genome shotgun sequence	AAGCTTACAGGGAGAAAGCTATACTCTCAATCCTGGGTACTGGTAGTAACCTTG CAAGAAGTCACCCCTTCGTACAGGCGTCCCTGAGATCCGCCACGGCCGACACAAG AAGATGTGCAGCTCCCTGATGTTGACGCCCCAGTACACAGTACCGAGTAGTTCA CCTGGCTGGAGAGGCTGCATTTTCTGGTTATAAGCTTCCCAACTTTCTTGTACAA AGTTGTCCCCGAATTC	236	77
No. 8	PREDICTED: <i>Apis florea</i> uncharacterized protein LOC100865969	AAGCTTCGTGGATCCAGATATCCTGCAGGAATTTCCCAACTGGGTGAGACCGTTAG TGAAAGAACGTGCTGAGTGTGGTTTCAACGCCTTAACGATTGGAGGCATTACTTT ATCAAGACTCGTGTCAAACGCAACAAGAATTGGCAGAAATATTGTCATTTCAAAT CGCCTTAGAGTTATGGGAATGATTTCAGAAACAAGGAAATTGAGTACCGTACAAGT AGAAGCCAAGAGATGTTGAACAGCTGCCTGCAAGGTAAAGACGGAAGGAATTC	273	80
No. 9	<i>Linepithema humile</i> nuclear hormone receptor E75, transcript variant X5, mRNA	AAGCTTTTAATCGTGCCAAAGGCTCTCTACCTTGATTACGTTGGGCGCTGGAGACG GCGACCTCTTGGACAGATTCAACGGTTGCTGAGAGTCCGCGGTGTCTACTTGTAG TTCCATCATGTTGGGAACCGCGACGTCCTCATTATCGTCGAATGGGGCTGGAT GACGATCTCGACGAAGGTGTAGGTGAGGTAATCATAAGGAATTC	209	83
No. 10	<i>Diaphorina citri</i> glutamine synthetase 2 cytoplasmic-like, transcript variant X4, mRNA	GAATTCATAGCATATTGCCCTCTACGTTGCCTGTCACACTAAAGGCCCAACAAC ACCACAAGAGCCTTGATCCTTGACTTTGGCAACGGCCCTTTCTCCCTCCAATCG AAAGAATCGGGCAACTTGATATCGGGTATATCGGCCTGCATAAACTAAGCTCGTT CTCATTCTTAAGCTCGCTTCTGTAGCCCAACCCCTTGGAGAATTCTTCCCTAGTC AAATCTGCGAATTTGGTGGGTCCGTACACCAGGTAAACCGAAGCTT	265	74
No. 11	<i>Tarenaya hassleriana</i> cysteine proteinase. RD21a-like, transcript variant X2, misc_RNA	GAATTCATAGCATATTGCCCTCTACGTTGCCTGTCACACTAAAGGCCCAACAAC ACCACAAGAGCCTTGATCCTTGACTTTGGCAACGGCCCTTTCTCCCTCCAATCG AAAGAATCGGGCAACTTGATATCGGGTATATCGGCCTGCATAAACTAAGCTCGTT CTCATTCTTAAGCTCGCTTCTGTAGCCCAACCCCTTGGAGAATTCTTCCCTAGTC AAATCTGCGAATTTGGTGGGTCCGTACACCAGGTAAACCGAAGCTT	268	84
No. 12	<i>Callithrix jacchus</i> Y box binding protein 3 (YBX3), mRNA	GAATTCCTGTACTATGTACGCGCCTGCTGATCGGACAACCTTTTCTTGGGTTATTC TTAGTGATAGCAGACTGATGTACAAATACGTCTTCTTTGGTATCATTACGATTGAT AAATCCATAGCCACTCTTGACGTTGACGTTGAACCATTTACAGTTCCAGTAACCTTTGTT GCTATCACTTCTTTATTTTTCACCGTCGTTTGTGGATTGCGGTTGTTCAATCGGCTG TGATTCCGCCGTTTCAGCCATTATTCAGATGTTGGTGGGCCCTTGGTACGTAAAA GCTT	282	83
No. 13	<i>Bombyx mori</i> glutamate dehydrogenase, mRNA	GAATTCATGATTCAATTCCTCTCTCTCTGTTCCAAAGTCAACTTAGATCCCCTAA CTTTACCCAGATCTTCAACAAGTTTATCTTCAACTAACATACAAGCCCTGTGGAA GAAATATTCCACCATGTGCGAAGAACCCTCGGACTTGCAATTTGTTGGCATATCTTTCA GTTTTTCCGGAATCTTGTGGGCATTCCTGGTCTGGAGGCTAAAGCTT	214	84
No. 14	<i>Prunus mume</i> extensin-2-like, mRNA	AAGCTTCATGGTGGAGATCCCGTGGAGGCCCTAGAAATGGTTATGGTGGTGGAC GCGATTTTGGATCATACGCGGCTGGTGGTGACAGATACGGTGGAGGCGGTTATGA TAGACCTCCAAGAGATTTTGGACCTGGTGGTGGCTTTGGAGGAGGAAGAGGCGG TTTTGCTGGAGGAGACAGGCGACCCTTCAATAACAGCTTCGGAGATCGTGGATCC CGGTCATTTGGTGAAGAAAGAGGAGGAGGCGGCTTTGAAGACAGAAGAGGAGGT TTTGAAGATAGACGACACCCATGGGGGTGATAGAAGACCTCCAATGATGGATC AACCTGGTCCAGGTGGTCCACGTGGAGGTGCTTCAGGGGGCTTTGACAGAACGG GAGATTTATATAGCAGAAGGGATAATGGACCCAAGGGTGGAGATTATAATGGTGG TCCAAATGGTTACAGTAATGGAGGATACGAGGGTGGTCCCGGCGCGGTTGGTTTT GGTGGCGGTGATCGTTACGGAGGCGGTCAAGCTGGTGGATACGGAGGCGATAGA GGAAAGTGGACTCTAAGGGAATTC	570	80
No. 15	<i>Spirometra erinaceieuropaei</i> genome assembly S_ erinaceieuropaei, scaffol	AAGCTTGACACACGTGTCTAAGGGGACCCTTTGGTATAAGGGACTGTTCTTTTTT TTTTTTTTTGTCAATCAACAATTTATCCAAGTAGGCTATTCCACATGTTGATTTTC TTGGACTCGGATGTACGCTGGTAGCTATAATCTCCCACGTGGTAAGGTCAATTG CTGCTGTAACCACAAATGCACGACTGACTTCTAATGTTGATTTCAATTTGTTGTTA TTTCCTTCAATGTTTCTCATTCTAACACTTCTCATCGTATTGCACAGGGCCGATAA CAGTAGTGAAAGCCTCCCAACTTTCTTGTACAGAGTTGTCCCCGAATTC	327	90

No. 16	<i>Apteryx australis mantelli</i> genome assembly AptMant0, scaffold scaffold39	AAGCTTAAACTCGCCCCCCCCCTTAAACCAAGGCCTAAATATGACGAGGAAATCGT TATCAAACGAAGAGTGATAGAGGAGCCAAAAGATACTGGTCGCAAGGAACGCGTC AGGAGAAGGGCTCCAATCGAGGTTGAAATGACCAACAGAAAAGTCCCTCCTCGC GAAGTGTTTCAGACCCAGAAGAGTGCCTAATGGAAGACCCTTTGAAACGCTCTTGAA GAGGAGAGGCGGAAGAGAGAAGAAGAGGAAAAGAAGAGACTCAAAAGTAGAGC TGAAGGAAAGATGCTGAAGGCAGAGGCGAACATAGACAAACTGAGGGCAACAAT CAGAAAGAAAGCTAAGGACTTATCCATTATTTCCCAACTTTTGAATTC	374	84
No. 17	<i>Bacillus thuringiensis serovar finitimus</i> YBT-020 plasmid pBMB26, complete sequence	AAGCTTTTCGTTTATGTTTCATTGATACCCAGCCCGGAGTGACACAACCAAGTGAT CTGCAAAGTGAAAAGAACTTGATAATGGCTCAGCAAGCAGCTTTCTTCGAATATG CTGCCGCTCTGATTTCTATGAACGCTTCTTCTAGTGTCTATAAAAGTGGAGTGATA AGGCCAATGCCTGTGTATGCCGAGTGCTTTGTATCCTACGCCACCGTGTCAACCTG TATCGATGCCTGTGCAATATTCCTTTTGCTTACCTAGCAATGTACGTACTATAGATT ATAAGACTGGGCAATGAATTC	299	93
No. 18	<i>Heligmosomoides polygyrus</i> genome assembly H_bakeri_Edinburgh, scaffold HPBE_contig0014130	AAGCTTTCTATAGACCTTTTTTCCGTCTCTTAATTGCATTCTCCCCAAGGGTTCCC TAGTAGCTTCGTTGTCTTCAATTTCTCTCCTCCTTCCGATACTTCTATTTCTTCC TCTTCATACTCAACATCCGATTCTTCCTCAGACTCGTCTTTGTCTTCTTCTTATT TTCGATTTCTATATCTCTTGTGTAAACGTTTATAACGGAGCCTTGGTTAGAACCAG ACAAAACGGTACTTCCACTATCTTCGCTCGATGACTTAGCGCTCGAATTC	274	79
No. 19	<i>Parascaris equorum</i> genome assembly P_equorum, scaffold PEQ_scaffold0036355	AAGCTTTATTTAAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATC AGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGG ACCTCCACTCACTTGGACGACGCTGCCGTGCTGACAAAGACGATCCATTAAGGAG CGAGTCTTCTCCTCGTACTTTCGCTCGCCCCATCGCTGCGGGCTAAATCCGAGC CCGGGGCCGAACCCCTTAGCACACCCCTCCCCACATAACTTATTTTCGACCGTATAT ATGGGGGGGAAATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTTCGTT AGCCTGACCGAAGATGAGGTGTTGGGGTAACAGCATCCATTGTCTCACGTGCGT GGGGGAACGTGAGTTATTTACGGGGGTGGCTGGTATGAATTC	486	96
No. 20	<i>Megachile rotundata</i> nucleosome assembly protein 1-like 1, transcript variant X2, mRNA	AAGCTTGTTTTCTCGCGGTGTAGTACGAACAGTTTCAGAAAAGTGTGCGCAATGAC TCATTCTTCAACTTCTTCGCTCCTCCAGTTGTTCCCGAAGATAGTAAGGAGGATG AAGTTGACGATGAAATTCACCAGATCTTGACGACTGATTTTGAATCGGTCATTAT ATCAGAGAGCGTATCATTCCTAGGGCGGTTCTATACTTCACTGGCGAAGGAATTG AAGATGAAGAAGAAGATTTTGAAGAGGAAGAAGATGATGATGATGAGGAGGGGT CCTAGCGGAATTC	288	74
No. 21	<i>Tribolium castaneum</i> microtubule-actin cross-linking factor 1 isoform X9	AAGCTTCTTGACCGTTTGGTGGAAGCTCAACAAGACCGGAGAGGCGCTAGTCA GACTCTGCACCGAAGACGATGGCGCCAAGGTACAGGAACCTCGGACAGCGACA ACGATAGATACGCGCTCTGAAGGCTGAGCTCAGGCAACGCCAACAAGCCCTCGA AGATGCCCTCCAGGAGAGCTCACAGTTCAGCGACAACTGGAAGGTATGCTGCG AGCCCTGTCCAACACTGCCGACACTGAATTC	248	75
No. 22	<i>Xenopus (Silurana) tropicalis</i> dedicator of cytokinesis 1 (dock1), transcript variant X2, mRNA	AAGCTTTATTTAAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATC AGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGG ACCTCCACTCACTTGGACGACGCTGCCGTGCTGACAAAGACGATCCATTAAGGAG CGAGTCTTCTCCTCGTACTTTCGCTCGCCCCATCGCTGCGGGCTAAATCCGAGC CCGGGGCCGAACCCCTTAGCACACCCCTCCCCACATAACTTATTTTCGACCGTATAT ATGGGGGGGAAATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTTCGTT AGCCTGACCGAAGATGAGGTGTTGGGGTAACAGCATCCATTGTCTCACGTGCGT GGGGGAACGTGATGTTATTTACGAGGGTTGGCTGGTATGAATTC	487	96
No. 23	<i>Harmonia axyridis</i> clone DS_3_034 18S ribosomal RNA gene, partial sequence	AAGCTTCCCCAACTTTGTACAAGAAAGTTGGGGAGCTTATCTTGCTTGTGGCCG ACGTTCCACAGAGAGAATTCCCAACTCAAGAGTCCCTCCACGTAGTTCTTCTCTTG CTGTCTGTACTTCCCTGCTCCCTTGCAAGCAGATTTCAGAGCCCTCTTCTCTGAAG TCTTGGAACCTTCATGACTTCTGCCATGATGAAACCTTTCTCGAAGTCGGTATGGA TGCGACCTGCAGCTTGTGGCGCCTTCGTACCTTTTGTATCGTCCAAGCTTGCGT CGAGGGCACTCCATTCCCCGTTACCCGTAACAACCATGGTAGGCGCAGAACCTAC CATCGACAGTTGATAAGGCAGACATTTGAAAGATGCGTCGCCGTTACGAGACCAT GCGATCAGCTTAAAGTTATTCAGAGTCACCAAATTTGTACGATGACGAGCGAACCC GCCACCTATTTGGTTTTGTATCTAATAAAAGCGCTCCTTCCGTTCCCGTCCGGAGCT CTATTTGCATGTATTAGCTCTAGAATTC	523	100
No. 24	<i>Biomphalaria glabrata</i> (chitinase-like protein PB1E7.04c, partial mRNA	AAGCTTGCCCAACCAAGCCCAACCAACACAGCAAGACCAACAACCCCAAGTAGTAC TACAACAACAGCAAAACCTACAACAAAACCATCGACGACAGCAAAACCAACAACCT CCTTCAACTACTACAACAACAGCAGCAGCACTCCAGTACCGTCTCTCCACTACTA CTACGAAGCCAACCAACCAACCCCTGCGCCAAATCCTAGCAAAACCTGATGTGGG AACCTGGAATGTTACAGGAATTC	243	71

RNAi of *H. axyridis* ecdysone receptor A

T-Blunt vector cloning

After amplification of *H. axyridis* ecdysone receptor A with the primers generated from GenBank accession number AB506666.1, a band was observed at approximately 200 bp (Fig. 3). Considering that the predicted size of the PCR product was 233 bp, this result indicates that the ecdysone receptor A was successfully amplified.

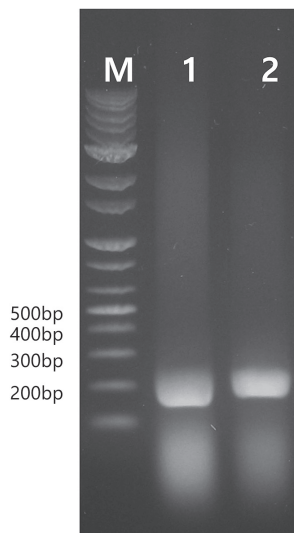


Fig. 3. Electrophoresis of ecdysone receptor A and B1 PCR products. A major band of approximately 200 bp was identified. M: 1-kb DNA ladder marker; Lane 2: PCR products amplified with EcRA FW; Lane 3: PCR products amplified with EcRB1 FW.

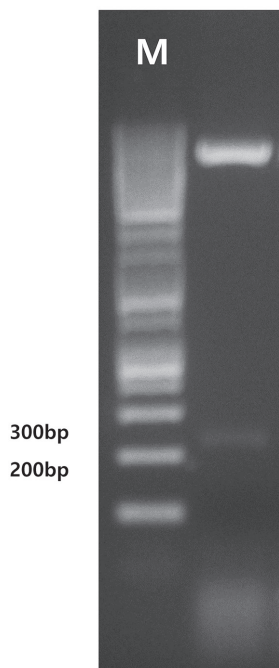


Fig. 4. Electrophoresis of plasmid DNA digested with *EcoRI* and *HindIII*. Two bands were detected: one of approximately 1 kb corresponding to the T-Blunt vector, and one of 233 bp corresponding to ecdysone receptor A. M: 1-kb DNA ladder marker; Lane 1: plasmid DNA cut with *EcoRI* and *HindIII*.

After cloning *H. axyridis* ecdysone receptor A into a T-Blunt vector, plasmids were digested with *EcoRI* and *HindIII* to confirm the presence of the insert within the vector. Consistent with the previous PCR results, a band of approximately 200 bp in size was observed (Fig. 4).

LITMUS 28i vector cloning and dsEcRA synthesis

After cloning *H. axyridis* ecdysone receptor A into the LITMUS 28i vector, plasmid DNA was digested with *EcoRI* and *HindIII* to confirm insertion. We accordingly observed a band of approximately 200 bp corresponding to the ecdysone receptor A insert (Fig. 5). The LITMUS 28i vector harboring the cloned ecdysone receptor A was digested with *HpaI* so that it could be used as a template for synthesis of dsRNA. We accordingly obtained a 28i vector: ecdysone receptor A product of approximately 3.5 kb (Fig. 5).

Double-stranded ecdysone receptor A synthesis

Ecdysone receptor A dsRNA was synthesized using linearized DNA as a template. A band corresponding to the synthesized dsRNA was accordingly observed at approximately 400–500 bp (Fig. 6).

Changes in phenotype after the injection with double-stranded ecdysone receptor A

After injection of the 4th-instar larvae of *H. axyridis* with dsEcRA or distilled water, those larvae injected with dsEcRA were of smaller size and in some individuals there was a lack of pigment expression in the outer cuticle of the thorax compared with those individuals injected with distilled water and those in the non-treatment group (Fig. 7). Furthermore, most of the 4th-instar larvae injected with dsEcRA had died within 4 days of injection, whereas some did not pupate but remained at the prepupal stage (Table 4).

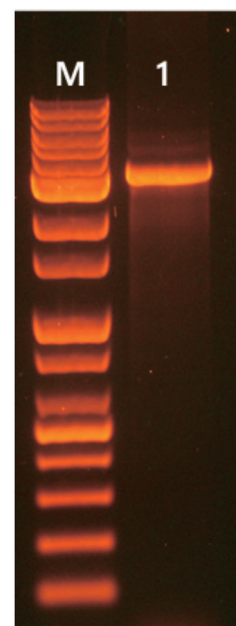


Fig. 5. Electrophoresis of plasmid DNA digested with *HpaI*. A 28i vector: ecdysone receptor A band was observed between 3 kb and 4 kb. M: 1-kb DNA ladder marker; Lane 1: plasmid DNA cut with *HpaI*.

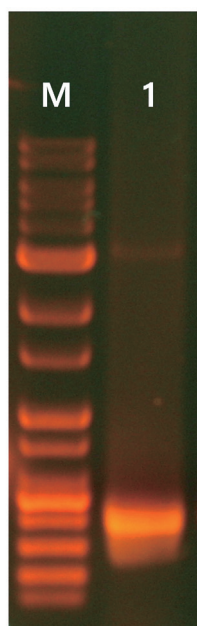


Fig. 6. Electrophoresis of double-stranded RNA. A band corresponding to the synthesized double-stranded ecdysone receptor A (dsEcRA) was observed in the region 400 bp to 500 bp. M: 1-kb DNA ladder marker; Lane 1: The synthesized dsEcRA between 400 bp and 500 bp.

Expression analysis of *H. axyridis* ecdysone receptor A

Real-time PCR was conducted to analyze the expression level of ecdysone receptor A following injection of injected to the 4th-instar larvae of *H. axyridis*. We observed that the expression level of ecdysone receptor A gradually started to decrease at day 3 after dsEcRA injection and that there was hardly any expression on day 4. In contrast, compared with the dsEcRA treatment group, there was a high level of ecdysone receptor A expression in the non-treatment and distilled water treatment groups (Fig. 8).

DISCUSSION

H. axyridis, which is widely used as a natural enemy for control of aphids, is characterized by having elytra of various colors and patterns. Komai (1956) suggested that the elytral color patterns vary depending on single and multiple allele genes. In the present study, to determine the genes involved in determining the colors and patterns of *H. axyridis* elytra, a cDNA library was generated and the functions of genes were analyzed using RNA interference (RNAi). RNAi technology has similarly been used in a number of previous studies to ana-

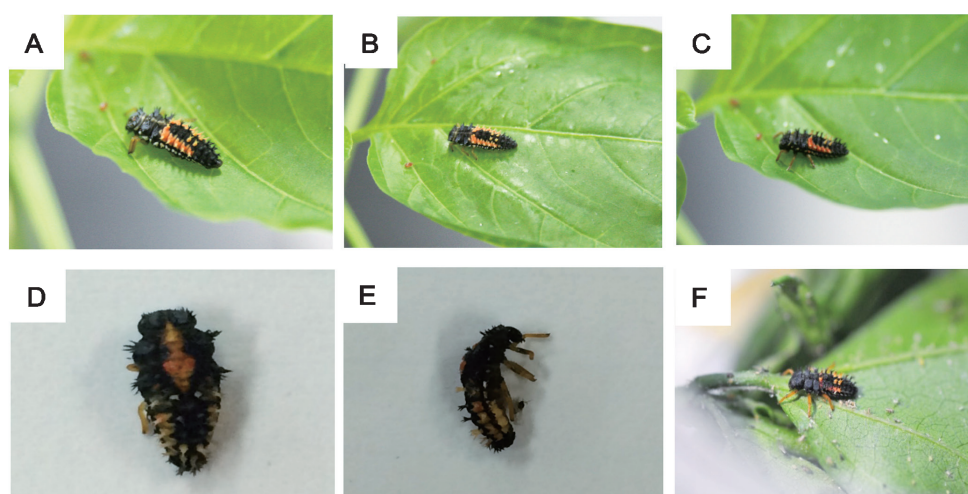


Fig. 7. Phenotype observed after injection of double-stranded ecdysone receptor A (dsEcRA.) (A) Untreated control; (B) Phenotype of a *Harmonia axyridis* imago obtained from a 4th-instar larva injected with distilled water; (C) Phenotype of an *H. axyridis* imago obtained from a 4th-instar larva injected with dsEcRA; (D, E) EcRA RNAi caused severe developmental defects and most *H. axyridis* larvae did not develop beyond the pupal stage and died; (F) An abnormal individual that failed to complete ecdysis with a lack of integument color.

Table 4. Effect of injecting 4th-instar *Harmonia axyridis* larvae with double-stranded ecdysone receptor A on adult phenotype

Injection	No. of injected larvae	No. of dead larvae	No. of dead pupae	No. of dead adults	No. of normal adults
Non-injection	240	0	0	0	240
DW	151	26	0	0	125
dsEcRA	180	164	16	0	0

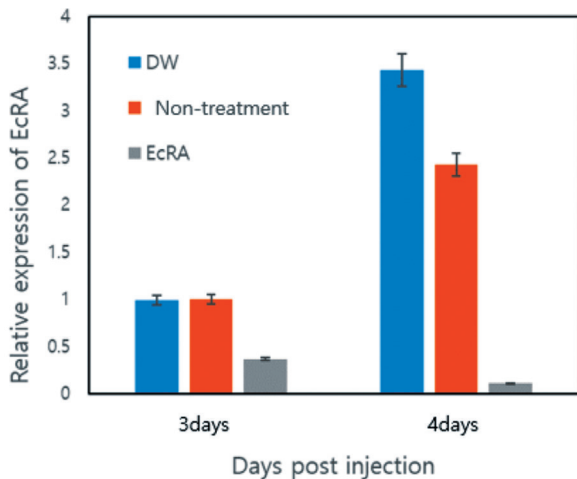


Fig. 8. Reduction in the relative abundance of ecdysone receptor A (EcRA) transcript in *Harmonia axyridis* injected with double-stranded (ds) RNA. *H. axyridis* larvae were injected with 3 μ l of distilled water or dsRNA (1 μ g/insect) of EcRA. *H. axyridis* were collected at 3 and 4 days post-injection and gene expression of the EcRA transcript was analyzed by real-time quantitative RT-PCR relative to the distilled water-injected insects, using *H. axyridis* ribosomal protein 49 (rp49) as the internal reference gene. Each bar represents the mean and standard error (SE) of the mean of two experimental replicates for three independent biological replicates at each time point.

lyze gene functions in insects. For example, injecting the eggs of *H. axyridis* expressing enhanced green fluorescent protein (EGFP) with dsEGFP reduced expression of the former (Kuwayama *et al.*, 2006), and injection with Distal-less and Arista-less dsRNA inhibited the development of legs and antennae in *H. axyridis* (Niimi *et al.*, 2005). This shows that RNAi in larvae can be effective for analyzing imago development in *H. axyridis*. In the present study, restriction enzyme sites for *Eco*RI and *Hind*III were inserted in adapter sequences during the generation of a cDNA library so that the genes in the library could easily be cloned into the LITMUS 28i transcription vector. When the sizes of genes in the completed cDNA library of *H. axyridis* were analyzed, inserts between approximately 200 and 700 bp were identified, which, excluding the size of the adapter sequences attached to both ends of the cDNA, were determined to be approximately 100–500 bp in size (Fig. 2). In *Diabrotica undecimpunctata howardi*, a high lethality rate of 95% was observed when the size of dsRNA used in RNAi was between a minimum size of 60 bp and a maximum size 240 bp (Bolognesi *et al.*, 2012). This indicates that knock-down occurs effectively in the insect body when the size of dsRNA is 60 bp or larger. Furthermore, random sequencing of 24 DNAs followed by NCBI BLAST searches, conducted to determine the genetic information of the completed cDNA library of *H. axyridis*, showed that most of the sequences were from insect genes and one of them was from an *H. axyridis* gene (Table 3). Due to the lack of genetic analysis on *H. axyridis* to date, most of the genes were identified as

genes of other insect species.

Before analyzing the function of genes in the generated *H. axyridis* cDNA library, ecdysone receptor A was knocked down and the inhibition of ecdysis was examined in order to validate the RNAi system using a transcription vector. Ecdysteroid (20-hydroxyecdysone), a hormone that plays crucial roles in insect development, reproduction, and immunity, binds to two nuclear receptors, ecdysone receptor (EcR) and ultraspiracle (USP) (Koelle *et al.*, 1991; Robertson *et al.*, 1993; Thomas *et al.*, 1993; Yao *et al.*, 1993). EcR has three nuclear hormone receptor isoforms, EcR-A, EcR-B1, and EcR-B2 (Koelle *et al.*, 1991; Talbot *et al.*, 1993). EcR proteins occur in larval or pupal tissues and also in the ovary and nervous system of imagoes (Carney and Bender, 2000; Dalton *et al.*, 2009; Robinow *et al.*, 1993; Schubiger *et al.*, 1998; Talbot *et al.*, 1993). In *H. axyridis*, EcR has two nuclear hormone receptor isoforms, EcR-A and EcR-B1 (Minakuchi *et al.*, 2014). In the present study, one of these receptors, ecdysone receptor A, was synthesized as dsRNA using in vitro transcription, and was subsequently injected into the abdomen of 0 to 2-day-old 4th-instar larvae of *H. axyridis*. Most of the larvae died within 4 days after injection at the 4th-instar stage, and some did not show the expression of pigments in the outer cuticle of the thorax (Fig. 8). In addition, the mRNA expression level of *H. axyridis* ecdysone receptor A showed a marked decrease on day 4 after dsRNA injection. In *Tribolium castaneum*, injection of larvae with dsRNA when they had just entered the late-instar stage inhibited their development and they failed to pupate (Tan and Palli, 2008). Furthermore, when dsBgEcR-A was injected into 6th-instar nymphs of *Blattella germanica*, most of the imagoes lacked extended fore wings and hind wings, with the aberration being more severe in the hind wings (Cruz *et al.*, 2006). Moreover, knock down of BgEcR-A prevented ecdysis to the imago stage. Inhibition of ecdysis has also been observed in a number of other insects subjected to RNAi, including *Drosophila melanogaster* and *Manduca sexta* (Cruz *et al.*, 2006; Dai and Gilbert, 1997; Gilbert, 1991; Romana *et al.*, 1995).

Our results show that the RNAi system mediated through dsRNA injection is an effective technique in *H. axyridis*. We anticipate that the *H. axyridis* cDNA library generated in this study will contribute to the functional analysis of *H. axyridis* genes via RNAi in further studies.

AUTHOR CONTRIBUTIONS

Yu Bin Jung designed the study, performed the comprehensive experiments, analyzed the data and wrote the paper. Jeong Hee Kim participated in cDNA library construction. Hye Ri Kwon performed the mass-culturing of the multicolored Asian ladybird beetle. Hyoun Sub Lim supervised to gateway cloning system. Yong Man Yu edited the paper. Chisa Yasunaga-Aoki participated in the design of the study and discussed the experiments and the results. Young Nam Youn supervised the

work and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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REFERENCES

- Amdam, G. V., Z. L. Simões, K. R. Guidugli, K. Norberg and S. W. Omholt 2003 Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotech.*, **3**: 1–8
- Arakane, Y., D. G. Hogenkamp, Y. C. Zhu, K. J. Kramer, C. A. Specht, R. W. Beeman, M. R. Kanost and S. Muthukrishnan 2004 Characterization of two chitin synthase genes of the red flour beetle, *Tribolium castaneum*, and alternate exon usage in one of the genes during development. *Insect Biochem. Mol. Biol.*, **34**: 291–304
- Aronstein, K. and E. Saldivar 2005 Characterization of a honey bee Toll related receptor gene *Am18w* and its potential involvement in antimicrobial immune defense. *Apidologie*, **36**: 3–14
- Attardo, G. M., S. Higgs, K. A. Klingler, D. L. Vanlandingham and A. S. Raikhel 2003 RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito *Aedes aegypti*. *Proc. Nat'l Acad. Sci.*, **100**: 13374–13379
- Bautista, M. A. M., T. Miyata, K. Miura and T. Tanaka 2009 RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, *Plutella xylostella*, reduces larval resistance to permethrin. *Insect Biochem. Mol. Biol.*, **39**: 38–46
- Belicek, J. 1976 Coccinellidae of Western Canada and Alaska with analyses of the transmontane zoogeographic relationship between the fauna of British Columbia and Alberta. *Quaest. Entomol.*, 283–409
- Bellés, X. 2010 Beyond *Drosophila*: RNAi in vivo and functional genomics in insects. *Ann. Rev. Entomol.*, **55**: 111–128
- Blandin, S., L. F. Moita, T. Köcher, M. Wilm, F. C. Kafatos and E. A. Levashina 2002 Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene. *EMBO Reports.*, **3**: 852–856
- Bolognesi, R., P. Ramaseshadri, J. Anderson, P. Bachman, W. Clinton, R. Flannagan, O. Ilagan, C. Lawrence, S. Levine, W. Moar, G. Mueller, J. Tan, J. Uffman, E. Wiggins, G. Heck and G. Segers 2012 Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diatraea virgifera virgifera* LeConte). *PLoS One.*, **7**: e47534.
- Brown, M. and S. Miller 1998 Coccinellidae (Coleoptera) in apple orchards of eastern West Virginia and the impact of invasion by *Harmonia axyridis*. *Entomol. News.*, **109**: 143–151
- Brown, S. J., J. P. Mahaffey, M. D. Lorenzen, R. E. Denell and J. W. Mahaffey 1999 Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. *Evol. Dev.*, **1**: 11–15
- Bucher, G., J. Scholten and M. Klingler 2002. Parental RNAi in *Tribolium* (Coleoptera). *Curr. Biol.*, **12**: R85–R86
- Carney, G. E. and M. Bender 2000 The *Drosophila ecdysone receptor (EcR)* gene is required maternally for normal oogenesis. *Genetics.*, **154**: 1203–1211
- Chapin, J. B. and V. Brou 1991 *Harmonia axyridis* (Pallas), the third species of the genus to be found in the United States (Coleoptera: Coccinellidae). *Proc. Entomol. Soc. Wash.*, **93**: 630–635
- Chen, X., H. Tian, L. Zou, B. Tang, J. Hu and W. Zhang 2008 Disruption of *Spodoptera exigua* larval development by silencing chitin synthase gene A with RNA interference. *Bull. Entomol. Res.*, **98**: 613–619
- Cruz, J., D. Mané-Padrós, X. Bellés and D. Martín 2006 Functions of the ecdysone receptor isoform-A in the hemimetabolous insect *Blattella germanica* revealed by systemic RNAi in vivo. *Dev. Biol.*, **297**: 158–171
- Dai, H., L. Ma, J. Wang, R. Jiang, Z. Wang and J. Fei 2008 Knockdown of ecdysis-triggering hormone gene with a binary UAS/GAL4 RNA interference system leads to lethal ecdysis deficiency in silkworm. *Acta Biochim. Biophys. Sin.*, **40**: 790–795
- Dai, J. D. and L. I. Gilbert 1997 Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. *Insect Biochem. Mol. Biol.*, **27**: 69–78
- Dalton, J. E., M. S. Lebo, L. E. Sanders, F. Sun and M. N. Arbeitman 2009 Ecdysone receptor acts in fruitless-expressing neurons to mediate drosophila courtship behaviors. *Curr. Biol.*, **19**: 1447–1452
- Day, W. H., D. R. Prokrym, D. R. Ellis and R. J. Chianese 1994 The known distribution of the predator *Propylea quatuordecimpunctata* (Coleoptera: Coccinellidae) in the United States, and thoughts on the origin of this species and five other exotic lady beetles in eastern North America. *Entomol. News.*, **105**: 244–256
- Dong, Y. and M. Friedrich 2005 Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper. *BMC Biotech.*, **5**: 25
- ESK 1994 Check list of insects from Korea (eds). *Kon-Kuk Univ. Press*, pp. 744
- Farooqui, T., K. Robinson, H. Vaessin and B. H. Smith 2003 Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J. Neurosci.*, **23**: 5370–5380
- Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nat.*, **391**: 806–811
- Gatehouse, H., L. Gatehouse, L. Malone, S. Hodges, E. Tregidga and J. Todd 2004 Amylase activity in honey bee hypopharyngeal glands reduced by RNA interference. *J. Apicul. Res.*, **43**: 9–13
- Gilbert, L. I. 1991 Metamorphosis of the *corpus allatum* and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Dev. Biol.*, **144**: 309–326
- Grbić, M., T. Van Leeuwen and R. M. Clark 2011 The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nat.*, **479**: 487–492
- Grill, C. P. and A. J. Moore 1998 Effects of a larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle. *Oecologia.*, **114**: 274–282
- Hossain, M., S. Shimizu, M. Matsuki, M. Imamura, S. Sakurai and M. Iwami 2008 Expression of 20-hydroxyecdysone-induced genes in the silkworm brain and their functional analysis in post-embryonic development. *Insect Biochem. Mol. Biol.*, **38**: 1001–1007
- Huang, J. H. and H. J. Lee 2011 RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female *Blattella germanica*. *J. Insect Physiol.*, **57**: 858–864
- Iperti, G. 1986 Les coccinellidae de France. *Phytoma.*, **377**: 14–22
- Iperti, G. 1999 Biodiversity of predaceous coccinellidae in relation to bioindication and economic importance. *Agricul. Ecosys. Environ.*, **74**: 323–342
- Jaubert-Possamai, S., G. Le Trionnaire, J. Bonhomme, G. K. Christophides, C. Rispe and D. Tagu 2007 Gene knockdown by RNAi in the pea aphid *Acyrtosiphon pisum*. *BMC Biotech.*, **7**: 63
- Kang, E. J., C. W. Jo, C. R. Park, K. S. Yoon, M. A. Kang, H. R. Kwon, M. J. Seo, Y. M. Yu and Y. N. Youn 2009 Effects of environmental factors on elytra colored patterns of multicolored Asian lady beetles, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Korean J. Appl. Entomol.*, **48**: 459–466
- Kennerdell, J. R. and R. W. Carthew 1998 Use of dsRNA-mediated genetic interference to demonstrate that frizzled and *friz-*

- zled 2 act in the wingless pathway. *Cell.*, **95**: 1017–1026
- Khila, A., M. Grbić 2007 Gene silencing in the spider mite *Tetranychus urticae*: dsRNA and siRNA parental silencing of the Distal-less gene. *Dev. Genes Evol.*, **217**: 241–251
- Kidd, K., C. Nalepa, E. and Day, M. Waldvogel 1995 Distribution of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) in North Carolina and Virginia. *Proc. Entomol. Soc. Wash.*, **97**: 729–731
- Koelle, M. R., W. S. Talbot, W. A. Segraves, M. T. Bender, P. Cherbas and D. S. Hogness 1991 The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell.*, **67**: 59–77
- Komai, T. 1956 Genetics of lady beetles. *Adv. Gen.*, **8**: 155–188
- Korschefsky, R. 1932 Coccinellidae. Pages In: Schenkling S, editor. *Coleopterorum Catalogus*, 439–447
- Kumar, M., G. P. Gupta and M. V. Rajam 2009 Silencing of acetylcholinesterase gene of *Helicoverpa armigera* by siRNA affects larval growth and its life cycle. *J. Insect Physiol.*, **55**: 273–278
- Kuwayama, H., T. Yaginuma, O. Yamashita and T. Niimi 2006 Germ-line transformation and RNAi of the ladybird beetle, *Harmonia axyridis*. *Insect Mol. Biol.*, **15**: 507–512
- Lamana, M. L. and J. C. Miller 1998 Temperature-dependent development in an Oregon population of *Harmonia axyridis* (Coleoptera: Coccinellidae). *Environ. Entomol.*, **27**: 1001–1005
- Lee, J. H. and T. J. Kang 2004 Functional response of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) to *Aphis gossypii* Glover (Homoptera: Aphididae) in the laboratory. *Biol. Con.*, **31**: 306–310
- Lynch, J. A. and C. Desplan 2006 A method for parental RNA interference in the wasp *Nasonia vitripennis*. *Nat. Protoc.*, **1**: 486–494
- Mackauer, M. 1976 Genetic problems in the production of biological control agents. *Ann. Rev. Entomol.*, **21**: 369–385
- Martin, D., M. D. Piulachs and X. Belles 1995 Patterns of haemolymph vitellogenin and ovarian vitellin in the German cockroach, and the role of juvenile hormone. *Physiol. Entomol.*, **20**: 59–65
- Minakuchi, C., T. Yokoi, S. Takimoto, A. Hosoda, M. Akamatsu, H. Tamura and Y. Nakagawa 2014 cDNA cloning of ecdysone receptor (EcR) and ultraspiracle (USP) from *Harmonia axyridis* and *Epilachna vigintioctopunctata* and the evaluation of the binding affinity of ecdysone agonists to the in vitro translated EcR/USP heterodimers. *J. Pest. Sci.*, **39**: 76–84
- Misquitta, L. and B. M. Paterson 1999 Targeted disruption of gene function in *Drosophila* by RNA interference (RNAi): a role for nautilus in embryonic somatic muscle formation. *Proc. Natl. Acad. Sci.*, **96**: 1451–1456
- Miyawaki, K., T. Mito, I. Sarashina, H. Zhang, Y. Shinmyo, H. Ohuchi and S. Noji 2004 Involvement of Wingless/Armadillo signaling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatus* (Orthoptera), as revealed by RNAi analysis. *Mech. Dev.*, **121**: 119–130
- Moriyama, Y., T. Sakamoto, S. G. Karpova, A. Matsumoto, S. Noji and K. Tomioka 2008 RNA interference of the clock gene period disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. *J. Biol. Rhythms.*, **23**: 308–318
- Nalepa, C., K. Kidd and K. Ahlstrom 1996 Biology of *Harmonia axyridis* (Coleoptera: Coccinellidae) in winter aggregations. *Ann. Entomol. Soc. Am.*, **89**: 681–685
- Niimi, T., H. Kuwayama and T. Yaginuma 2005 Larval RNAi applied to the analysis of postembryonic development in the ladybird beetle, *Harmonia axyridis*. *J. Insect Biotechnol. Sericol.*, **74**: 95–102
- Ohnishi, A., J. J. Hull and S. Matsumoto 2006 Targeted disruption of genes in the *Bombyx mori* sex pheromone biosynthetic pathway. *Proc. Natl. Acad. Sci. USA.*, **103**: 4398–4403
- Price, D. R. and J. A. Gatehouse 2008 RNAi-mediated crop protection against insects. *Trends Biotechnol.*, **26**: 393–400
- Quan, G., T. Kanda and T. Tamura 2002 Induction of the white egg 3 mutant phenotype by injection of the double-stranded RNA of the silkworm white gene. *Insect Mol. Biol.*, **11**: 217–222
- Rajagopal, R., S. Sivakumar, N. Agrawal, P. Malhotra and R. K. Bhatnagar 2002 Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *J. Biol. Chem.*, **277**: 46849–46851
- Robertson, N., G. Schulman, S. Karnik, E. Alnemri and G. Litwack 1993 Demonstration of nuclear translocation of the mineralocorticoid receptor (MR) using an anti-MR antibody and confocal laser scanning microscopy. *Mol. Endocrinol.*, **7**: 1226–1239
- Robinow, S., W. S. Talbot, D. S. Hogness and J. W. Truman 1993 Programmed cell death in the *Drosophila* CNS is ecdysone regulated and coupled with a specific ecdysone receptor isoform. *Develop.*, **119**: 1251–1259
- Romana, I., N. Pascual and X. Belles 1995 The ovary is a source of circulating ecdysteroids in *Blattella germanica* (Dictyoptera: Blattellidae). *Euro. J. Entomol.*, **92**: 93–93
- Sakai, T., Y. Uehara and M. Matsuka 1974 The effect of temperature and other factors on the expression of elytral pattern in lady beetle, *Harmonia axyridis* Pallas. *Bull. Fac. Agricul. Tamagawa Univ.*, **14**: 33–39
- Schubiger, M., A. A. Wade, G. E. Carney, J. W. Truman and M. Bender 1998 *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Develop.*, **125**: 2053–2062
- Suzuki, Y., J. W. Truman and L. M. Riddiford 2008 The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa. *Develop.*, **135**: 569–577
- Tabunoki, H., S. Higurashi, O. Ninagi, H. Fujii, Y. Banno, M. Nozaki, M. Kitajima, N. Miura, S. Atsumi and K. Tsuchida 2004 A carotenoid-binding protein (CBP) plays a crucial role in cocoon pigmentation of silkworm (*Bombyx mori*) larvae. *FEBS Letters.*, **567**: 175–178
- Talbot, W. S., E. A. Swyryd and D. S. Hogness 1993 *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell*, **73**: 1323–1337
- Tan, A. and S. R. Palli 2008 Ecdysone receptor isoforms play distinct roles in controlling molting and metamorphosis in the red flour beetle, *Tribolium castaneum*. *Mol. Cell. Endocrinol.*, **291**: 42–49
- Tedders, W. and P. Schaefer 1994 Release and establishment of *Harmonia axyridis* (Coleoptera: Coccinellidae) in the southeastern United States. *Entomol. News.*, **105**: 228–243
- Thomas, H. E., H. G. Stunnenberg and A. F. Stewart 1993 Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nat.*, **362**: 471–475
- Tomoyasu, Y. and R. E. Denell 2004 Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. *Develop. Genes Evol.*, **214**: 575–578
- Turner, C., M. Davy, R. MacDiarmid, K. Plummer, N. Birch and R. Newcomb 2006 RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. *Insect Mol. Biol.*, **15**: 383–391
- Watanabe, C. 1964 A tentative catalogue of insect natural enemies of injurious insects in Japan – Part 1. Parasite–Predator Host Catalogue (compiled by Yasumatsu, K. and C. Watanabe). *Ent. Lab. Fac. Agri. Kyushu Univ.*, 166 pp
- Yao, T. P., B. M. Forman, Z. Jiang, L. Cherbas, J. D. Chen, M. McKeown, P. Cherbas and R. M. Evans 1993 Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nat.*, **366**: 476–479