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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  $4.43 \times 10^4$  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 1<sup>st</sup> and 2<sup>nd</sup> sections of the abdomen of 0 to 2-day-old 4<sup>th</sup>-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) <sub>25</sub>	Biotin-GGG GAC AAC TTT GTA CAA GAA AGT TGG GAA GCT T(N) <sub>22</sub>
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) <sub>22</sub> 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  $\mu$ l of cDNA, 10  $\mu$ l of 5 $\times$  Adapter Buffer, 5  $\mu$ l of 5' Adapter Mix (0.5  $\mu$ g/ $\mu$ l), 8  $\mu$ l of 0.1M DTT, and 5  $\mu$ l of T4 DNA Ligase (1 U/ $\mu$ l) were mixed together to give a total volume of 50  $\mu$ l, which was then reacted at 16°C for 20 h.

To 79  $\mu$ l of first-strand cDNA with attached attB1 adapter, we added 10  $\mu$ l of 10 $\times$  High Fidelity PCR buffer, 4  $\mu$ l of 10 mM (each) dNTPs, 5  $\mu$ l of 50 mM MgSO<sub>4</sub>, 1  $\mu$ l of Second-strand synthesis Primer (100 ng/ $\mu$ l) (Table 1), 1  $\mu$ l of High Fidelity Platinum<sup>®</sup> Taq DNA Polymerase, and 5  $\mu$ l of distilled water (total volume 100  $\mu$ 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<sup>®</sup> 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  $\mu$ 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<sup>®</sup> Top10 Electrocomp<sup>™</sup> *Escherichia coli* (Invitrogen, USA) using an electroporator. The electroporation conditions were as follows: voltage, 1.8 kV; resistance, 200  $\Omega$ ; and capacity, 25  $\mu$ F. The *E. coli* cells were incubated in S.O.C media for 1 h. The resulting transformants were serially diluted from 10<sup>-1</sup> to 10<sup>-4</sup> and 100- $\mu$ 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  $\mu$ 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  $\mu$ l of DEPC-treated water. Using the extracted total RNA as a template, cDNA was generated using ReverTra Ace- $\alpha$ -<sup>®</sup> 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- $\mu$ l reaction mixture, containing 3  $\mu$ l of cDNA, 2  $\mu$ l of 10 $\times$  Reaction Buffer, 2  $\mu$ l of 10 mM dNTP mixture, 1  $\mu$ l of HaEcRA FW and HaEcRA RV primers, 0.2  $\mu$ l of Prime Taq DNA polymerase, and 10.8  $\mu$ 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<sup>™</sup> PCR Cloning Kit (SolGent, Korea). Four microliters of PCR product, 1  $\mu$ l of 6 $\times$  T-Blunt buffer, and 1  $\mu$ l of T-Blunt vector were added to 1.5-ml Eppendorf tubes and the 6  $\mu$ l total reaction volume was maintained at room temperature overnight.

One microliter of cloning sample was used to transform *E. coli* (DH5 $\alpha$ ) competent cells. The cells were incubated for 1 h and 300- $\mu$ l aliquots of the resulting transformants were subsequently plated on Luria-Bertani (LB) plates containing kanamycin (1  $\mu$ 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  $\mu$ l) containing 8  $\mu$ l of plasmid DNA, 0.5  $\mu$ l of *Eco*RI, 0.5  $\mu$ l of *Hind*III, and 1  $\mu$ 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<sup>®</sup> PCR & Gel Purification Kit (Bioneer, Korea). *H. axyridis* ecdysone receptor A (25  $\mu$ l) was then cloned into the LITMUS 28i vector using a 10- $\mu$ l reaction mixture, containing 2  $\mu$ l of LITMUS 28i vector, 6  $\mu$ l of ecdysone receptor A DNA, 1  $\mu$ l of 10 $\times$  Ligase Buffer, and 1  $\mu$ l of T4 DNA Ligase (Promega, USA), which was incubated at room temperature overnight.

The cloned sample was used to transform *E. coli* (DH5 $\alpha$ ) competent cells. The cells were incubated for 1 h and 300- $\mu$ l aliquots of the resulting transformants were plated onto LB plates containing ampicillin (1  $\mu$ 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  $\mu$ l) containing 8  $\mu$ l of plasmid DNA, 0.5  $\mu$ l of *Eco*RI, 0.5  $\mu$ l of *Hind*III,

and 1  $\mu$ 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  $\mu$ l) containing 8  $\mu$ l of plasmid DNA, 1  $\mu$ l of *Hpa*I (Takara, Japan), and 1  $\mu$ 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  $\mu$ l of 200-fold diluted RNaseA and 1  $\mu$ 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  $\mu$ l DEPC-treated water.

#### Double-stranded ecdysone receptor A injection

Thirty to forty 4<sup>th</sup>-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 1<sup>st</sup> and 2<sup>nd</sup> sections of abdomen of the 4<sup>th</sup>-instar larvae. Larvae in the distilled water group were injected with 3  $\mu$ l of distilled water, whereas those in the dsEcRA group were injected with 1  $\mu$ 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 4<sup>th</sup>-instar larvae with dsEcRA. On days 2, 3, and 4 after injection, three of the 4<sup>th</sup>-instar larvae were selected from each treatment group, and total RNA was extracted using Trizol reagent. First-strand cDNA was synthesized from 1  $\mu$ g of total RNA using ReverTra Ace- $\alpha$ -® (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- $\mu$ l reaction mixtures containing 2  $\mu$ l of 1/5-fold diluted cDNA, 10  $\mu$ l of iQ™ SYBR® Green supermix, 0.5  $\mu$ l of forward primer, 0.5  $\mu$ l of reverse primer (Table 1), and 7  $\mu$ 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.

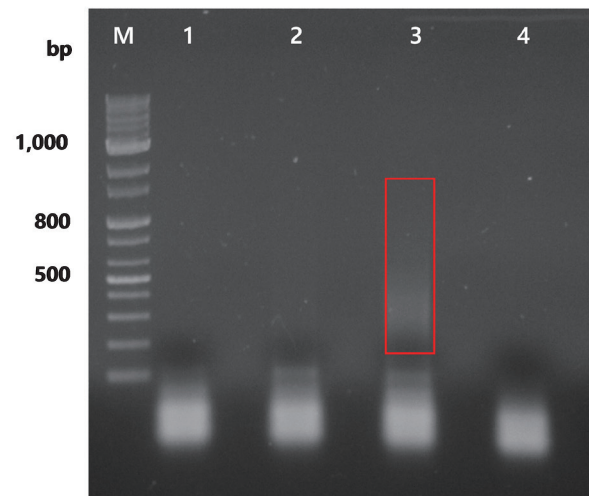
## RESULTS

### 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<sup>-3</sup> plate, 38 colonies on the 10<sup>-2</sup> plate, and 527 colonies on the 10<sup>-1</sup> plate. Finally, an *H. axyridis* cDNA library with a titer of 4.43  $\times$  10<sup>4</sup> was obtained (Table 2).

To confirm the size of genes in the *H. axyridis* cDNA library, 24 random colonies were selected from the 10<sup>-1</sup> 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).

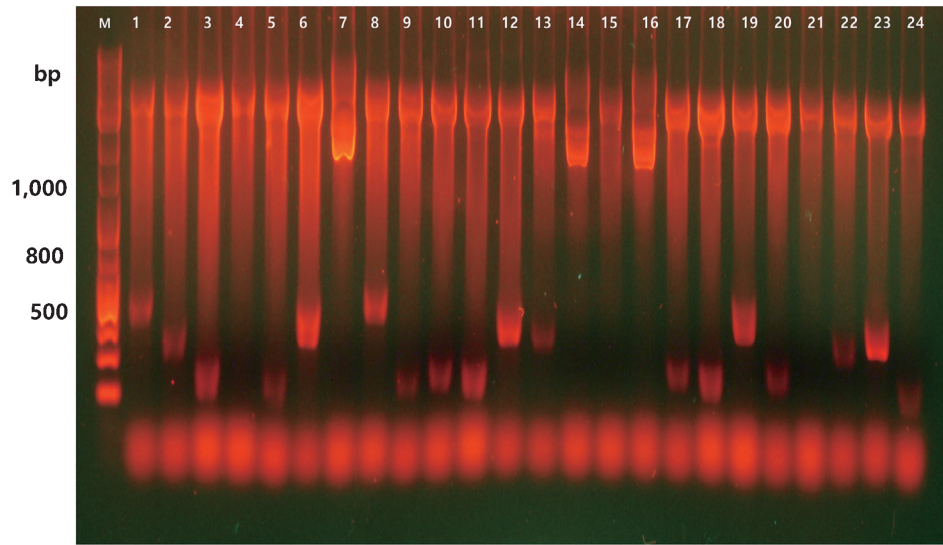


**Fig. 1.** Electrophoresis of *Harmonia axyridis* cDNA amplified using an N<sub>22</sub> random primer and a 5' primer. M: 1-kb DNA ladder marker; Lane 1: PCR product from 20 cycles; Lane 2: PCR product from 25 cycles; Lane 3: PCR product from 30 cycles; Lane 4: Negative control.

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

Dilution	Amount Plated ( $\mu$ l)	Colonies per Plate	Titer* (cfu/ml)	Average Titer* (cfu/ml)	Total Volume (ml)	Total CFUs (cfu)
10 <sup>-3</sup>	100	2	2 $\times$ 10 <sup>5</sup>			
10 <sup>-2</sup>	100	38	3.8 $\times$ 10 <sup>5</sup>	3.69 $\times$ 10 <sup>5</sup>	12	3.07 $\times$ 10 <sup>4</sup>
10 <sup>-1</sup>	100	527	5.27 $\times$ 10 <sup>5</sup>			

\* 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	AAGCTTGTGGAAACCTTCCCTGTTTCATGTCGTAGTTGGCGAAATGAGTGGACATG AGTAAGTATTCAAAGTTCTTGATGATGTGGGTTAGGGCGAATTTATTCAAGTCATC CTCGAGGTGACCAATGGCTCCATAGCGGGAGTTACAAGAAGATTCCGTGTTGGTG GTGCTTTGTTTATTGATATTTGGTCTGCAAGCATCTTGGTAGCCGTGAGACATTCT TGAGAATTC	232	78
No. 2	<i>Salmo salar</i> clone Contig 4326 Cystatin-B putative mRNA, complete cds	AAGCTTAACTTTGCCCTGGAGTCGACCACCGCAAATGTTTCTAAGTCAATTGAT TTTACATCTTTTGTATCATGTTGTTATATTTGATTTGACTTAAAAACCCCTTTTGTAT TGACGCGACTTTTGTATGTAACGCCACCGTGCAGACAAGAACCGAATAACTTGAG GAACTCAGACCCGATGTCGTGCGGGACAACCTTTGTACAAAAAGTTGGGAATTC	221	100
No. 3	<i>Sesamum indicum</i> putative late blight resistance protein homolog R1A-10, mRNA	AAGCTTTCCATACTTCTAAAGGTCCAAAAACAAGCTTTGCGACAACGGAGATGGTTF AGGGGGGTCCGAAAAGTGTCAATTTCCAAAATCACCTGTGTCTCTTGAACGGA AGCAGTTGTGCAAAATCTGATTTGGACCAACTTGAGTTTACGAAAAAGTAGGACA GGAGCGTGAAAGCCGCAAGGCTCTATCTTAGGTAACAGGCGAGAAAACCTGGCTG TCTCACCCAAAATGGGATGCGAATTGGAATTC	251	93
No. 4	<i>Tribolium castaneum</i> trithorax group protein osa, mRNA	AAGCTTTTTCGATAGCATCGTCCTGGGTGTCAGGTCTGCCGCCAGTCCGCAGCATCA GGGTTTTCCGAGTAGGCCCGCCAGCCTACCACACCTAATGCCCATGGACCGGAC GCAGCTGATCTGAGTGGGGCAATAGCAACGACAGTTCCGGTGGCCCGATCCCG GGAACGCCTAACTCGCAAGGTATGAGGCGGACCCCTCTCCAACAGGTTCAACGG GGTCCAGGTCGATGTCGCCGGCAGTTGGTCAACAGAACATTTCCCATGCCACCTCG ACCGTCCAGCGGGCCATCAGACCCTCCTACCAGAATCAGCCACTCTCCCATGGT TCTGCGCCAGGCAGCTACCCAAGTACACACCCTGCACCGCATCCCTCCCATGCGC CTCACGGGTACAAAAAGCATCCAGGGATGACACCATCGGCTCCTCCACCGCGCA ACAGATGACTATATATCAGACCAGCCAACAGTAACTTTGAGAATTC	486	76
No. 5	<i>Tribolium castaneum</i> nuclear transcription factor Y subunit B-4, transcript variant X3, mRNA	AAGCTTTTTTCGATAAGTAGATCACGGCAGAAAATACAGATGATTCGAATCACGA CGGCGGTGATAAACGTGGAGCACCTTTGAGAGAACAAGATAGATTTCTGCCAATT GCAAATGTAGCAAAAATTATGAAGCGTGCCATTCTGACACAGGAAAAGGTAAATA GCAAAATAGCAAAAAGATGCTAGGGGGTGTGTTCAAGAATGCGTTTCAGAGTTTAT CAGTTTTATAACTAGTGAAGCAAGTGATCGTTGTCATATGAAAAAAGGAAGACC ATAAACGGAGAGAACCATGAGAATTC	221	75

No. 6	<i>Tribolium castaneum</i> hypothetical protein TcasGA2_TC014392	AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA TCAAAATAAATTAAGATAACACATTAGACATTAATCTAAGCTCTACCCTTAAACTG CTTGTGTAATCTGGAAGTGAATCATCATACTGGGCTTGGAAAAAGTTACCAGCT ACTGCATTCGCCAAATTAATACTTTTCAGCGAACTTTTTTCACCGAAAAATTAACCCCT ATCCCATCGGTCTTGGAGTGTCTCGTTTCTTGAAGTCGACCTTACCAGGCTGTT TGAACAGCAGAAAGATACCTGTGAAGACCGGTATCTTAGGTGGCCCTGATCC CACGTACTCGTTCAATACTTCCCCTTTGGATAAATCAGAACGGGAATATTCACCAC CAGCCAATGTTGGAATTCGGGGACAACCTTGTGTACGAAGTTATATTTCAATTTGGTC AGACAAAATTGATTGACCAAAACCCCCATAACAGCAAATTTCTGCCGTGAG TATGAGCGCGAAAGCATTATAGGTGCGTTTCGGAGCGAGTTAGACGGTCTTACTA ACTCAGTTCCCCTTCTCTCATTTCGTGTAATTTCTTGTGTATTGTGTATGTGTCT TTGGGGTAGGCAAATTTGTGTTAGTTGTAAGGTGTTCTGATACCGATATAGC GATTATTATGTTAGGACATATGCATAATTTGAAAGACGATGGTTCCAATGTATGGT TAAACGCAGGCACCGGAAGCAACCAAATATATTAATTTGACAAAAGTGTACGAGA ACTTGGGATCATCTTTGTGTGCAAGTTTCCAGGATTTTCATGCATTAACCCGGAT GTGACTATAATCCTGCCTTTTTGAAATTC	859	77
No. 7	<i>Dendroctonus ponderosae</i> , whole genome shotgun sequence	AAGCTTACAGGGGAGAAAGACTATACTCTCAATCCTGGGTACTGGTAGTAACCTTG CAAGAAGTCACCCTTCGTACAGGCGTCCCTGAGATCCGCCACGGCCGACACAAG AAGATGTGCAGCTCCCTGATGTTGACGCCCCAGTACACACGTACCGAGTAGTTCA CCTGGCTGGAGAGGCTGCATTTTCTGGTTATAAGCTTCCAACCTTCTTGTACAA AGTTGTCCCGAATTC	236	77
No. 8	PREDICTED: <i>Apis florea</i> uncharacterized protein LOC100865969	AAGCTTCGTGGATCCAGATATCCTGCAGGAATTCCTCAACTGGGTGAGACCGTTAG TGAAAGAACGTGCTGAGTGTGGTTTCAACGCCCTAACGATTGGAGGCATTACTTT ATCAAGACTCGTGTCAAACGCAACAAGAATTTGGCAGAATTTATGTCATTTCAAAT CGCCTTAGAGTTATGGGAATGATTTCAGAAAAGGAAATTTGAGTACCGTACAAGT AGAAGCCAAGAGATGTTGAACAGCTGCCTGCAAGGTAAGACGGAAGGAATTC	273	80
No. 9	<i>Linepithema humile</i> nuclear hormone receptor E75 , transcript variant X5, mRNA	AAGCTTTTAATCGTGCCAAGGCTCTCTACCTTGATTACGTTGGGCGCTGGAGACG GCGACCTCTTGGACAGATTCAACGGTTGCTGAGAGTCCGCGGTGTCTACTTGTAG TTCCATCATGTTGGAAACCGGCGACGCTCATTATCGTCAATGGGGCTGGAT GACGATCTCGCAAGCTGTAGGTGAGGTAATCATAAGGAATTC	209	83
No. 10	<i>Diaphorina citri</i> glutamine synthetase 2 cytoplasmic-like, transcript variant X4, mRNA	GAATTCATAGCATATTTGCCCTCTACGTTGCCTGTACACTAAAGGCCCAACAACCT ACCACAAGAGCCTTGATCCTTGACTTTGGCAACGGCCCTTTCTCCCTCCAATCG AAAGAATCGGGCAACTTGATATCGGGTATATCGGCCTGCATAAACTAAGCTCGTT CTCATTCTAAGCTCGCTTCTGTAGCCCAACCCCTTGGAGAATTTCTCCCTAGTC AAATCTGCGAATTTGGTGGGTCCGTACACCAGGTAACCGAAGCTT	265	74
No. 11	<i>Tarenaya hassleriana</i> cysteine proteinase. RD21a-like, transcript variant X2, misc_RNA	GAATTCATAGCATATTTGCCCTCTACGTTGCCTGTACACTAAAGGCCCAACAACCT ACCACAAGAGCCTTGATCCTTGACTTTGGCAACGGCCCTTTCTCCCTCCAATCG AAAGAATCGGGCAACTTGATATCGGGTATATCGGCCTGCATAAACTAAGCTCGTT CTCATTCTAAGCTCGCTTCTGTAGCCCAACCCCTTGGAGAATTTCTCCCTAGTC AAATCTGCGAATTTGGTGGGTCCGTACACCAGGTAACCGAAGCTT	268	84
No. 12	<i>Callithrix jacchus</i> Y box binding protein 3 (YBX3), mRNA	GAATTCCTGTACTATGTACGCGCCTGTGATCGGACAACCTTTCTTGGGTTATTC TTAGTGATAGCAGACTGATGTACAAATACGCTCTTCTTTGGTATCATTACGATTGAT AAATCCATAGCCACTCTTGACGTTGAACCATTTACAGTTCCAGTAACTTTGTT GCTATCACTTCTTTATTTTTACCCTGCTTTTGGATTTCGGTTGTTCAATCGGCTG TGATTCGCCGTTTTCAGCCATTATTCAGATGTTGGTGGGCCCTTGGTACGTAAAA GCTT	282	83
No. 13	<i>Bombyx mori</i> glutamate dehydrogenase, mRNA	GAATTCATGATTCATTTCTCTCTCTCTGTTCCAAAGTCAACTTAGATCCCCTAA CTTTACCCAGATCTTCAACAAGTTTATCTTCAACTAACATACAAGCCCTGTGGAA GAAATATTCCACCATGTGGAAGAACCCTCGGACTTGCATTTGTTGGCATACTTTCA GTTTTTCCGGAATCTTGTGGGCATTCCTGGTCTGGAGGCTAAAGCTT	214	84
No. 14	<i>Prunus mume</i> extensin-2-like, mRNA	AAGCTTCATGGTGGAGATCCCGTGGAGGCCCTAGAAATGGTTATGGTGGTGGAC GCGATTTTGGATCATACGGCGGTGGTGGTGACAGATACGGTGGAGGCGGTTATGA TAGACCTCCAAGAGATTTTGGACCTGGTGGTGGCTTTGGAGGAGGAAGAGGCGG TTTTGCTGGAGGAGACAGGCCACCCTTCAATAACAGCTTCGGAGATCGTGGATCC CGGTCAATTTGGTGAAGAAAGAGGAGGAGGCGGCTTTGAAGACAGAAGAGGAGGT TTTGAAGATAGACGACCACCCATGGGGGTGATAGAAGACCTCCAATGATGGATC AACCTGGTCCAGGTGGTCCACGTTGGAGGTGCTTCAGGGGGCTTTGACAGAACGG GAGATTTATATAGCAGAAGGGATAATGGACCCAAGGGTGGAGATTATAATGGTGG TCCAAATGGTTACAGTAATGGAGGATACGAGGGTGGTCCCGCGCCGGTGGTTTT GGTGGCGGTGATCGTTACGGAGGCGGTCAAGCTGGTGGATACGGAGGCGATAGA GGAAAGTGGACTCTAAGGGAATTC	570	80
No. 15	<i>Spirometra erinaceieuropaei</i> genome assembly S_ erinaceieuropaei, scaffold	AAGCTTGACACACGTGTCTAAGGGGACCCTTTGGTATAAGGGACTGTTCTTTTT TTTTTTTTTGTCAATCAACAATTAATCCAAGTAGGCTATCCACATGTTGATTTTC TTGGACTCGGATGTACGCTGGTAGCTATAATCTCCCACGTGGTAAGGTCAATTTG CTGCTGTAACCACAATGCACGACTGACTTCTAATGTTGATTTCAATTTGTTGTTA TTTTCTTCAATGTTTCTATTCTAACACTTCTCATCGTATTGCACAGGGCCGATAA CAGTAGTGAAAGCCTCCAACCTTTCTTGTACAGAGTTGTCCCGAATTC	327	90

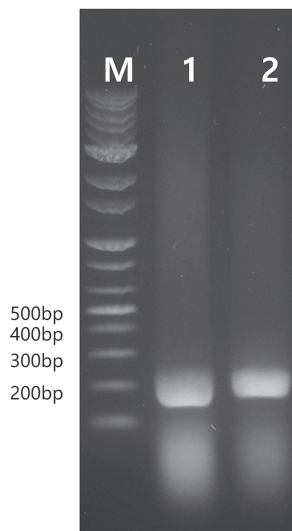
No. 16	<i>Apteryx australis mantelli</i> genome assembly AptMant0, scaffold scaffold39	AAGCTTAAACTCGCCCCCCTTAAACCAAGGCCTAAATATGACGAGGAAATCGT TATCAAACGAAGAGTGATAGAGGAGCCAAAAGATACTGGTTCGCAAGGAAACGCGTC AGGAGAAGGGCTCCAATCGAGGTTGAAATGCCAAACAGAAAAGTCCCTCCTCGC GAAGTGTTTCAGACCCAGAAAGAGTGCCTAATGGAAGACCCTTTGAAAACGCTTTGAA GAGGAGAGGCGGAAGAGAGAAGAAGAGGAAAAGAAGAGACTCAAAAGTAGAGC TGAAGGAAAGATGCTGAAGGCAGAGGCGAACATAGACAAAAGTGAAGGGCAACAAT CAGAAAGAAAGCTAAGGACTTATCCATTATTTCCCAACTTTTGAATTC	374	84
No. 17	<i>Bacillus thuringiensis serovar finitimus</i> YBT-020 plasmid pBMB26, complete sequence	AAGCTTTTCGTTTATGTTTCATTGATACCCAGCCCGGAGTGACACAACCAAGTGAT CTGCAAAGTGAAAAGAACTTGATAATGGCTCAGCAAGCAGCTTTCTTCGAATATG CTGCCGCTCTGATTTCTATGAACGCTTCTTCTAGTGTCTATAAAAGTGGAGTGATA AGGCCAATGCCTGTGATGCCGAGTGCTTTGTATCCTACGCCACCGTGTCAACCTG TATCGATGCCTGTGCAATATCTTTTGTCTTACCTAGCAATGTACGTACTATAGATT ATAAGACTGGGCATGAATTC	299	93
No. 18	<i>Heligmosomoides polygyrus</i> genome assembly H_bakeri_Edinburgh, scaffold HPBE_contig0014130	AAGCTTTCTATAGACCTTTTTTCCGCTCTCTTAATTGCATTTCTCCCAAGGGTTCCC TAGTAGCTTCGTTGTCTTCAATTTCTCTCCTCTTCCGATACTTCTATTTCTTCC TCTTCATACTCAACATCCGATTCTTCCCTCAGACTCGTCTTTGTCTTCTTCTTATT TTCGATTTCTATATCTTGTGTAAACGTTTATAACGGAGCCTTGGTTAGAACCAG ACAAAACGGTACTTCCACTATCTTCGCTCGATGACTTAGCGCTCGAATTC	274	79
No. 19	<i>Parascaris equorum</i> genome assembly P_equorum, scaffold PEQ_scaffold0036355	AAGCTTTATTTAAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCAGGGCGTTACTCGTGCCTTAGATGTATTGATCTACATGTGGAGTCATCCATC AGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGG ACCTCCACTCACTTGGACGACGCTGCCGTCGTGACAAAGACGATCCATTAAGGAG CGAGTCTTCCCTCCTGACTTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGC CCGGGGCCGAACCCCTTAGCACACCCCTTCCACATAACTTATTTGACCGTATAT ATGGGGGGGAAATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTTCGTT AGCCTGACCGAAGATGAGGTGTTGGGGTAACAGCATCCATTGTCTCAGTGCCT GGGGGAACGTGAGTTATTTACGGGGGTTGGCTGGTATGAATTC	486	96
No. 20	<i>Megachile rotundata</i> nucleosome assembly protein 1-like 1, transcript variant X2, mRNA	AAGCTTGTTTTCTCGCGGTGTAGTACGAACAGTTCAGAAAAGTGTGCGCAATGAC TCATTCTTCAACTTCTTCGCTCCTCCAGTTGTTCCCGAAGATAGTAAGGAGGATG AAGTTGACGATGAAATTCACCAGATCTTGACGACTGATTTTGAATCGGTCATTAT ATCAGAGAGCGTATCATTCTAGGGCGGTTCTATACTTCACTGGCGAAGGAATTG AAGATGAAGAAGAAGATTTTGAAGAGGAAGAAGATGATGATGATGAGGAGGGGT CCTAGCGGAATTC	288	74
No. 21	<i>Tribolium castaneum</i> microtubule-actin cross-linking factor 1 isoform X9	AAGCTTCTTGACCGTTTGGTGGCAAGCTCAACAAGACCGGAGAGGCGCTAGTCA GACTCTGCACCGAAGACGATGGCGCAAGGTACAGGAACCTCCTGGACAGCGACA ACGATAGATACGCGACTCTGAAGGCTGAGCTCAGGCAACGCCAACAAGCCCTCGA AGATGCCCTCCAGGAGAGCTCACAGTTCAGCGACAAAAGTGAAGGTATGCTGCG AGCCCTGTCCAACACTGCCGACACTGAATTC	248	75
No. 22	<i>Xenopus (Silurana) tropicalis</i> dedicator of cytokinesis 1 (dock1), transcript variant X2, mRNA	AAGCTTTATTTAAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCAGGGCGTTACTCGTGCCTTAGATGTATTGATCTACATGTGGAGTCATCCATC AGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGG ACCTCCACTCACTTGGACGACGCTGCCGTCGTGACAAAGACGATCCATTAAGGAG CGAGTCTTCCCTCCTGACTTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGC CCGGGGCCGAACCCCTTAGCACACCCCTTCCACATAACTTATTTGACCGTATAT ATGGGGGGGAAATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTTCGTT AGCCTGACCGAAGATGAGGTGTTGGGGTAACAGCATCCATTGTCTCAGTGCCT GGGGGAACGTGATGTTATTTACGAGGGTTGGCTGGTATGAATTC	487	96
No. 23	<i>Harmonia axyridis</i> clone DS_3_034 18S ribosomal RNA gene, partial sequence	AAGCTTCCCAACTTTGTACAAGAAAGTTGGGGAGCTTATCTTGTCTTGTGGCCG ACGTTCCACAGAGAGAATTCCCAACTCAAGAGTCCCTCCACGTAGTTCTTCCCTG CTGTCTGTACTTCCCTGCTCCCTTGCAAGCAGATTCAGAGCCCTCTTCCCTGAAG TCTTGGAACTTCATGACTTCTGCCATGATGAAACCTTTCTCGAAGTCCGTATGGA TGCGACCTGCAGCTTGTGGCGCCTTCGTACCTTTTGTATCGTCCAAGCTTGCCT CGAGGGCACTCCATTCCCGTTACCCGTAACAACCATGGTAGGCGCAGAACCCTAC CATCGACAGTTGATAAGGCAGACATTTGAAAGATGCGTCGCCGTTACGAGACCAT GCGATCAGCTTAAAAGTTATTCAGAGTACCAAATTTGTACGATGACGAGCGAACCC GCCACCTATTTGGTTTTGATCTAATAAAAAGCGCTCCTTCCGTTCCCGTCCGGAGCT CTATTTGCATGTATTAGCTCTAGAATTC	523	100
No. 24	<i>Biomphalaria glabrata</i> (chitinase-like protein PB1E7.04c, partial mRNA	AAGCTTGCCCAACCAAGCCCAACCAACAGCAAGACCAACAACCCCAAGTAGTAC TACAACAACAGCAAAACCTACAACAACCAACATCGACGACAGCAAAACCAACAAC CCTTCAACTACTACAACAACCAACAGCAGCAGCACTCCAGTACCCTCCACTACTA CTACGAAGCCAACCAACCAACCCCTGCGCCAAATCCTAGCAAACCTGATGTGGG AACCTGGAATGTTACAGGAATTC	243	71



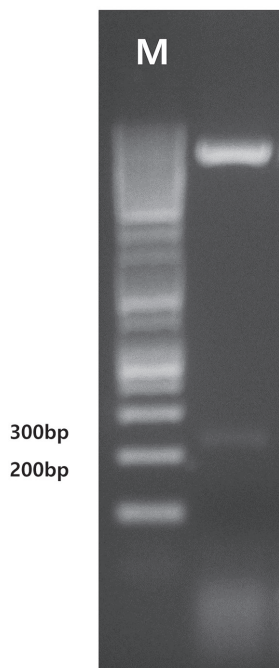
### RNAi of *H. axyridis* ecdysone receptor A

#### 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

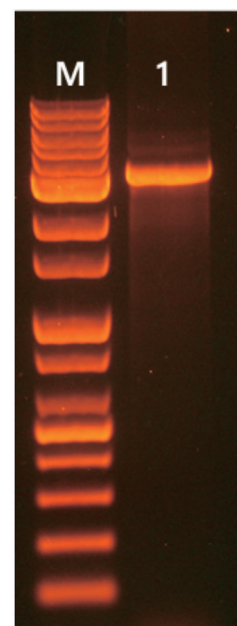
After cloning *H. axyridis* ecdysone receptor A into the LITMUS 28i vector, plasmid DNA was digested with *EcoRI* and *HindIII* to confirm insertion. We accordingly observed a band of approximately 200 bp corresponding to the ecdysone receptor A insert (Fig. 5). The LITMUS 28i vector harboring the cloned 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

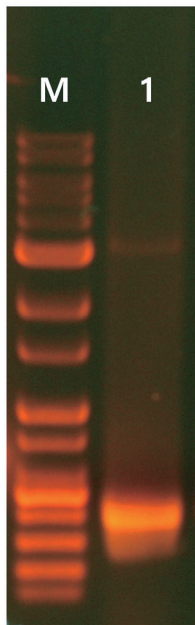
Ecdysone receptor A dsRNA was synthesized using linearized DNA as a template. A band corresponding to the synthesized dsRNA was accordingly observed at approximately 400–500 bp (Fig. 6).

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

After injection of the 4<sup>th</sup>-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 4<sup>th</sup>-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*.



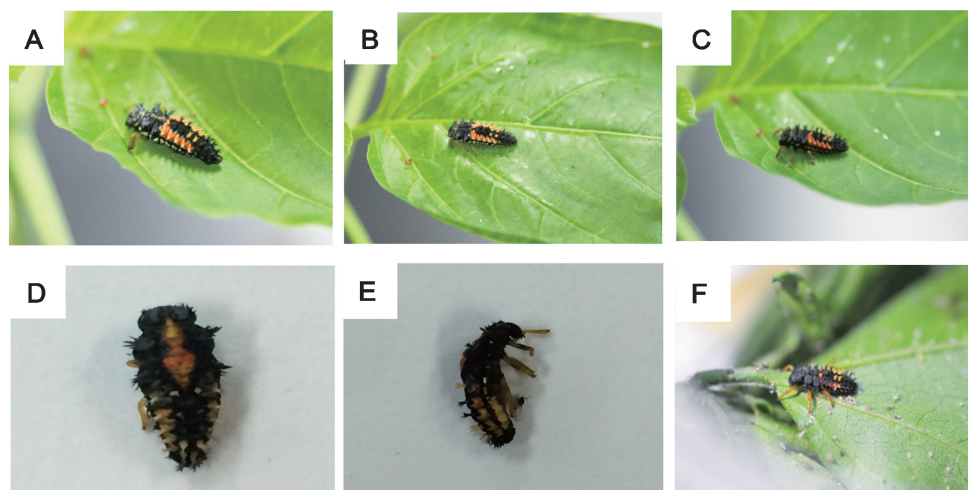
**Fig. 6.** Electrophoresis of double-stranded RNA. A band corresponding to the synthesized double-stranded ecdysone receptor A (dsEcRA) was observed in the region 400 bp to 500 bp. M: 1-kb DNA ladder marker; Lane 1: The synthesized dsEcRA between 400 bp and 500 bp.

#### 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 4<sup>th</sup>-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

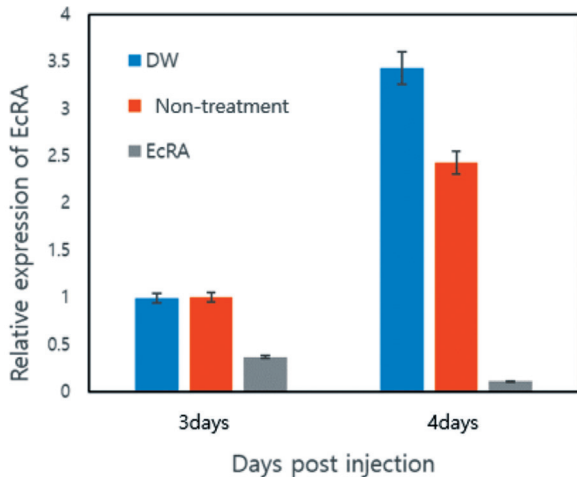
*H. axyridis*, which is widely used as a natural enemy for control of aphids, is characterized by having elytra of various colors and patterns. Komai (1956) suggested that the elytral color patterns vary depending on single and multiple allele genes. In the present study, to determine the genes involved 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 4<sup>th</sup>-instar larva injected with distilled water; (C) Phenotype of an *H. axyridis* imago obtained from a 4<sup>th</sup>-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 4<sup>th</sup>-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  $\mu$ l of distilled water or dsRNA (1  $\mu$ 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 4<sup>th</sup>-instar larvae of *H. axyridis*. Most of the larvae died within 4 days after injection at the 4<sup>th</sup>-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 6<sup>th</sup>-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.

#### ACKNOWLEDGEMENTS

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