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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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 (Korschensky, 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 (Belles, 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),...
dsRNA has been studied in various insects, including fruit flies (Kerenderell and Carthew, 1998), red flour beetle (Brown et al., 1999), Gambian mosquito (Blandin et al., 2004), silkworm moth (Ohnishi et al., 2004), German cockroach (Bellés, 2010; Huang and Lee, 2011; Moriyama et al., 2012), cricket (Morimaya et al., 2008), and two–spotted spider mite (Grbić et al., 2011; Khila and Grbić, 2007). Feeding of dsRNA has been studied in tritricid 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., 2009), 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 5X First–strand buffer, 5 µl of 0.1 M DTT, 2.5 µl of 10 mM (each) dNTPs, and 5 µl of SuperScript®II 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

<p>| First strand synthesis | Second strand synthesis | attB2–(N)15 | attB1–adapter–DOWN | TCG TCG GGG ACA ACT TTG TAC AAA AAA GGT GGG AAT TC |</p>
<table>
<thead>
<tr>
<th>Clones</th>
<th>5′–Oligo</th>
<th>5′–Oligo sequence</th>
<th>3′–Oligo</th>
<th>3′–Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB1–adapter–UP</td>
<td>TCG TCG GGG ACA ACT TTG TAC AAA AAA GGT GGG AAT TC</td>
<td>Biotin–GGG GAC AAC TTT GTA CAA GAA AGT TGG GAA GCT T(N)15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>attB1–adapter–DOWN</td>
<td>PGAA TTC CCA ACT TTT TTG TAC AAA GGT GTC CCC</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**DNA PCR**

<table>
<thead>
<tr>
<th>pDONR 207 F</th>
<th>TCG CGT TAA CGC TAG CAT GGA TCT C</th>
<th>pDONR 207 R</th>
<th>GTA ACA TCA GAG ATT TTG AGA CAC</th>
</tr>
</thead>
</table>

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An attB1 adapter was attached to the 5’ end of the first–strand cDNA (Table 1). Thereafter, 22 µl of cDNA, 10 µl of 5X Adapter Buffer, 5 µl of 5’ Adapter Mix (0.5 µg/µl), 8 µl of 0.9 M DTT, and 5 µl of T4 DNA Ligase (1 U/µl) were mixed together to give a total volume of 50 µl, which was then reacted at 16°C for 20 h. 

To 79 µl of first–strand cDNA with attached attB1 adapter, we added 10 µl of 10X High Fidelity PCR buffer, 4 µl of 10 mM each dNTPs, 5 µl of 50 mM MgSO4, 1 µl of Second–strand synthesis Primer (100 ng/µl) (Table 1), 1 µl of High Fidelity Platinum® Taq DNA Polymerase, and 5 µl of distilled water (total volume 100 µl), 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° 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 µl 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 µF. The *E. coli* cells were incubated in S.O.C media for 1 h. The resulting transformants were serially diluted from 10⁻¹ to 10⁴ and 100–µl aliquots were plated onto genta-mycin–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 µl, 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 µl 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 (GENETHIO, 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–µl reaction mixture, containing 3 µl of cDNA, 2 µl of 10X Reaction Buffer, 2 µl of 10 mM dNTP mixture, 1 µl of HaECR Fa and HaECR RV primers, 0.2 µl of Prime Taq DNA polymerase, and 10.8 µl of distilled water in a 0.2–m l 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 µl of 6X T–Blunt buffer, and 1 µl of T–Blunt vector were added to 1.5–ml Eppendorf tubes and the 6 µl total reaction volume was maintained at room temperature overnight.

One microliter of cloning sample was used to transform *E. coli* (DH5α) competent cells. The cells were incubated for 1 h and 300–µl aliquots of the resulting transformants were subsequently plated on Luria–Bertani (LB) plates containing kanamycin (1 µg/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 µl) containing 8 µl of plasmid DNA, 0.5 µl of *Eco*RI, 0.5 µl of *Hind*III, and 1 µl 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 µl) was then cloned into the LITMUS 28i vector using a 10–µl reaction mixture, containing 2 µl of LITMUS 28i vector, 6 µl of ecdysone receptor A DNA, 1 µl of 10X Ligase Buffer, and 1 µl of T4 DNA Ligase (Promega, USA), which was incubated at room temperature overnight.

The cloned sample was used to transform *E. coli* (DH5α) competent cells. The cells were incubated for 1 h and 300–µl aliquots of the resulting transformants were plated onto LB plates containing ampicillin (1 µg/ml) and incubated overnight. Single colonies grown on the ampicillin plates were 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 µl) containing 8 µl of plasmid DNA, 0.5 µl of *Eco*RI, 0.5 µl of *Hind*III,
and 1 µl of 2.1 buffer were incubated at 37ºC 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 µl) containing 8 µl of plasmid DNA, 1 µl of *Hpa*I (Takara, Japan), and 1 µl of 10×K Buffer were incubated at 37ºC 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 µl of 200–fold diluted RNaseA and 1 µl of undiluted DNase were added and reacted at 37ºC to remove residual DNA templates and ssRNA. After the reaction, the dsEcRA was concentrated and dissolved in 20 µl 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 µl of distilled water, whereas those in the dsEcRA group were injected with 1 µl 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 µg 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–µl reaction mixtures containing 2 µl of 1/5–fold diluted cDNA, 10 µl of iQ™ SYBR® Green supermix, 0.5 µl of forward primer, 0.5 µl of reverse primer (Table 1), and 7 µl of distilled water. The reactions were carried out under the following conditions: 95ºC for 3 min, 95ºC for 10 s, 58ºC for 10 s, and 72ºC for 20 s, which were repeated for 39 cycles, and then reacted at 95ºC for 10 s, 65ºC for 5 s, and 95ºC 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 × 10⁴ 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 N₂ 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.](image-url)

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

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Amount Plated (µl)</th>
<th>Colonies per Plate</th>
<th>Titer* (cfu/ml)</th>
<th>Average Titer* (cfu/ml)</th>
<th>Total Volume (ml)</th>
<th>Total CFUs (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻³</td>
<td>100</td>
<td>2</td>
<td>2×10⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻²</td>
<td>100</td>
<td>38</td>
<td>3.8×10⁶</td>
<td>3.69×10⁶</td>
<td>12</td>
<td>3.07×10⁷</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>100</td>
<td>527</td>
<td>5.27×10⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>EST blast search</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Identity (%)</th>
</tr>
</thead>
</table>
| **No. 1**
*Asobara tabida* hch gene for ferritin heavy chain and hch gene for ferritin light chain | AAGCTTTGTGGAAACCTTCCCTGTTCATGTCGTAGTTGGCGAAATGAGTGGACATGAGTAAGTATTCAAAGTTCTTGATGATGTGGGTTAGGGCGAATTTATTCAAGTCATCCTCGAGGTGACCAATGGCTCCATAGCGGGAGTTACAAGAAGATTCCGTGTTGGTG GTGCTTTTGGTTATGTGATTTTGGTCTGCAAGCATCTTGGTAGCCGTGAGACATTCTTGAGAATTC | 232 | 78 |
| **No. 2**
*Salmo salar* clone Contig 4326 Cystatin–B putative mRNA, complete cDNA | AAGCTTTACCTGCCCCCTGGAAGTGGCACCCGCACCAATGTTTCTAAGTCAATTGATTTTACATCTTTTGTATCATGTTGTTATATTGTATTGTACTTAAAAACCCTTTTGTATGACGCGACTTTTGATGTAAACGCCACCGTGCAGACAAGAACCGAATAACTTGAGGAACTCAGACCGCATGTCGTCGGGGACAACTTTGTACAAAAAAGTTGGGAATTC | 221 | 100 |
| **No. 3**
*Sesamum indicum* putative late blight resistance protein homolog R1A–10, mRNA | AAGCTTTTCTCCATACTTCTAAAAAGGTTTGGACAACGGGAGATGCTCAATTGATTTTACATCTTTTGTATCATGTTGTTATATTGTATTGTACTTAAAAACCCTTTTGTATGACGCGACTTTTGATGTAAACGCCACCGTGCAGACAAGAACCGAATAACTTGAGGAACTCAGACCGCATGTCGTCGGGGACAACTTTGTACAAAAAAGTTGGGAATTC | 251 | 93 |
| **No. 4**
*Tribolium castaneum* trithorax group protein osa, mRNA | AAGCTTTTCTCCATACTTCTAAAAAGGTTTGGACAACGGGAGATGCTCAATTGATTTTACATCTTTTGTATCATGTTGTTATATTGTATTGTACTTAAAAACCCTTTTGTATGACGCGACTTTTGATGTAAACGCCACCGTGCAGACAAGAACCGAATAACTTGAGGAACTCAGACCGCATGTCGTCGGGGACAACTTTGTACAAAAAAGTTGGGAATTC | 486 | 76 |
| **No. 5**
*Tribolium castaneum* nuclear transcription factor Y subunit B–4, transcript variant X3, mRNA | AAGCTTTTCTCCATACTTCTAAAAAGGTTTGGACAACGGGAGATGCTCAATTGATTTTACATCTTTTGTATCATGTTGTTATATTGTATTGTACTTAAAAACCCTTTTGTATGACGCGACTTTTGATGTAAACGCCACCGTGCAGACAAGAACCGAATAACTTGAGGAACTCAGACCGCATGTCGTCGGGGACAACTTTGTACAAAAAAGTTGGGAATTC | 221 | 75 |
No. 6 **Tribolium castaneum** hypothetical protein Tcga2_TCONS0002392

```
AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA
TCAAAATAAATTAAGATAACACATTAGACATTAATCTAAGCTCTACCCTTAAACTG
CTTGTGTAATTCTGGAACTGAGTCATCATACTGGGCTTGGAAAAAGTTACCAGCT
ACTGCATTCCCCAAATTATACTTTTACAGGAACCTCCCCAGCAGACATCCAGACAGCT
CCCTGGCTGGAAGCTACGTCATTTTTCTGGTTTTAAGATCTTCACCACCTTAAACCA
AGTGAACAGGAAGCTCCGATCAAGGACAAATATTATATGGCAAAAGTGAAGATAG
ACATTTGGGATCATCTTTGTGTCGAAGTTTGCCAGGATTTCATGCCATTAACCCGGAT
GTGACTATAATCCTGGTCTGCGAGTTTGGCAGAAGAGCTCCGTTTCTTCTGCTAC
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No. 7 **Dendroctonus ponderosae**, whole genome shotgun sequence

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AAGCTTACAGGGAGAAAGACTATACTCTCAATCCTGGGTACTGGTAGTAACCTTG
CAAGAAGTCACCCTTCGTACAGGCGTCCCTGAGATCCGCCCACGGCCGCCACAAG
AAGATGTGCAGCTCCCTGATGTTGACGCCCCAGTACACACGTACCGAGTAGTTCA
CCTGGCTGGAGAGGCTGCATTTTCTGGTTATAAGCTTCCCAACTTTCTTGTACAA
AGTTGTCCCCGAATTC
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No. 8 **Linepithema humile** nuclear hormone receptor E75, transcript variant X5, mRNA

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AAGCTTCGTGGATCCAGATATCCTGCAGGAATTCCCAACTGGGTGAGACCGTTAG
TGAAAGAACGTGCTGAGTGTGGTTTCAACGCCTTAACGATTGGAGGCATTACTTT
ATCAAGACTCGTGTCAAACGCAACAAGAATTGGCAGAATTATTGTCATTTCAAAT
CGCCTTAGAGTTATGGGAATGATTCAGAAACAAGGAAATTGAGTACCGTACAAGT
AGAAGCCAAGAGATGTTGAACAGCTGCCTGCAAGGTAAAGACGGAAGGAATTC
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No. 9 **Tarenaya hassleriana** cysteine proteinase. RD21a–like, transcript variant X2, misc_RNA

```
AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA
TCAAAATAAATTAAGATAACACATTAGACATTAATCTAAGCTCTACCCTTAAACTG
CTTGTGTAATTCTGGAACTGAGTCATCATACTGGGCTTGGAAAAAGTTACCAGCT
ACTGCATTCCCCAAATTATACTTTTACAGGAACCTCCCCAGCAGACATCCAGACAGCT
CCCTGGCTGGAAGCTACGTCATTTTTCTGGTTTTAAGATCTTCACCACCTTAAACCA
AGTGAACAGGAAGCTCCGATCAAGGACAAATATTATATGGCAAAAGTGAAGATAG
ACATTTGGGATCATCTTTGTGTCGAAGTTTGCCAGGATTTCATGCCATTAACCCGGAT
GTGACTATAATCCTGGTCTGCGAGTTTGGCAGAAGAGCTCCGTTTCTTCTGCTAC
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No. 10 **Callithrix jacchus** Y box binding protein 3 (YBX3), mRNA

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AAGCTTCGTGGATCCAGATATCCTGCAGGAATTCCCAACTGGGTGAGACCGTTAG
TGAAAGAACGTGCTGAGTGTGGTTTCAACGCCTTAACGATTGGAGGCATTACTTT
ATCAAGACTCGTGTCAAACGCAACAAGAATTGGCAGAATTATTGTCATTTCAAAT
CGCCTTAGAGTTATGGGAATGATTCAGAAACAAGGAAATTGAGTACCGTACAAGT
AGAAGCCAAGAGATGTTGAACAGCTGCCTGCAAGGTAAAGACGGAAGGAATTC
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No. 11 **Bombyx mori** glutamate dehydrogenase, mRNA

```
AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA
TCAAAATAAATTAAGATAACACATTAGACATTAATCTAAGCTCTACCCTTAAACTG
CTTGTGTAATTCTGGAACTGAGTCATCATACTGGGCTTGGAAAAAGTTACCAGCT
ACTGCATTCCCCAAATTATACTTTTACAGGAACCTCCCCAGCAGACATCCAGACAGCT
CCCTGGCTGGAAGCTACGTCATTTTTCTGGTTTTAAGATCTTCACCACCTTAAACCA
AGTGAACAGGAAGCTCCGATCAAGGACAAATATTATATGGCAAAAGTGAAGATAG
ACATTTGGGATCATCTTTGTGTCGAAGTTTGCCAGGATTTCATGCCATTAACCCGGAT
GTGACTATAATCCTGGTCTGCGAGTTTGGCAGAAGAGCTCCGTTTCTTCTGCTAC
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No. 12 **Prunus mume** extensin–2–like, mRNA

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AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA
TCAAAATAAATTAAGATAACACATTAGACATTAATCTAAGCTCTACCCTTAAACTG
CTTGTGTAATTCTGGAACTGAGTCATCATACTGGGCTTGGAAAAAGTTACCAGCT
ACTGCATTCCCCAAATTATACTTTTACAGGAACCTCCCCAGCAGACATCCAGACAGCT
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No. 13 **Bombyx mori** glutamate dehydrogenase, mRNA

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AAGCTTCGCGTTACTCGTTATAGCTCTTCTCAACAACTACAGCCCTACTTCAACAA
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CTTGTGTAATTCTGGAACTGAGTCATCATACTGGGCTTGGAAAAAGTTACCAGCT
ACTGCATTCCCCAAATTATACTTTTACAGGAACCTCCCCAGCAGACATCCAGACAGCT
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No. 14 **Prunus mume** extensin–2–like, mRNA

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ACTGCATTCCCCAAATTATACTTTTACAGGAACCTCCCCAGCAGACATCCAGACAGCT
CCCTGGCTGGAAGCTACGTCATTTTTCTGGTTTTAAGATCTTCACCACCTTAAACCA
AGTGAACAGGAAGCTCCGATCAAGGACAAATATTATATGGCAAAAGTGAAGATAG
ACATTTGGGATCATCTTTGTGTCGAAGTTTGCCAGGATTTCATGCCATTAACCCGGAT
GTGACTATAATCCTGGTCTGCGAGTTTGGCAGAAGAGCTCCGTTTCTTCTGCTAC
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No. 15 **Spirometra erinaceieuropaei** genome assembly S. erinaceieuropaei, scaffold
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<th>Query Coverage</th>
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<td>16</td>
<td><em>Apteryx australis</em> manielli assembly AptMant0, scaffold scaffold39</td>
<td>AAGCTTAAATCCTGCCCCCTTTTAAAAACAGGGCTAAATATGGAGGAGGGAATCTG TATCAGAAGGAGGCTAGTATGAGGACGAGCAGCAAAAGATCTGTCGCAAGAGGCAGCCTGAGAGAAGGCTCCAATCGAGGTTGAAATGACCAAACAGAACATGGACAGGACAGGACACATGGACAACTTCAGGCTACAGGGAGAAGGAGAGGAAAAGAAGAGACTCAAAAGTAGAGCTGAAGGAAAGATGCTGAAGGCAGAGGCGAACATAGACAAACTGAGGGCAACAATCAGAAACGATGACATCGTTATTTTCTGCTACAAATGATCTAGATTATAAGACTGGGCAAGATGAAATTC</td>
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<td>17</td>
<td><em>Bacillus thuringiensis</em> serovar finitimus YBT–020 plasmid pBMB26, complete sequence</td>
<td>AAGCTTTTATGAGACCTTTTTTTCCGCTTCTCTGCTTCGATCTACTTCTTTACCAGCTGCAATGACCAAACAGAAAAGTCCTCCTCGCGAAGTGTTCAGACCCAGAAGAGTGCCAATGGAAGACCCTTTGAAACGTCTTGAGAGGAGAGGCGGAAGAGAGAAGAAGAGGAAAAGAAGAGACTCAAAAGTAGAGCTGAAGGAAAGATGCTGAAGGCAGAGGCGAACATAGACAAACTGAGGGCAACAATCAGAAACGATGACATCGTTATTTTCTGCTACAAATGATCTAGATTATAAGACTGGGCAAGATGAAATTC</td>
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<td>93</td>
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<td>18</td>
<td><em>Heligmosomoides polygyrus</em> genome assembly H_bakeri_Edinburgh, scaffold HPBE_contig001430</td>
<td>AAGCTTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
<td>274</td>
<td>96</td>
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<tr>
<td>19</td>
<td><em>Parasarcis equorum</em> genome assembly P_equorum, scaffold PEQ_scaffold0036355</td>
<td>AAGCTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
<td>486</td>
<td>96</td>
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<tr>
<td>20</td>
<td><em>Megachile rotundata</em> nucleosome assembly protein 1–like 1, transcript variant X2, mRNA</td>
<td>AAGCTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
<td>288</td>
<td>74</td>
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<tr>
<td>21</td>
<td><em>Tribolium castaneum</em> microtubule–actin cross–linking factor 1 isoform X9</td>
<td>AAGCTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
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<td>22</td>
<td><em>Xenopus (Silurana) tropicalis</em> dedicator of cytokinesis 1 (dock1), transcript variant X2, mRNA</td>
<td>AAGCTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
<td>487</td>
<td>96</td>
</tr>
<tr>
<td>23</td>
<td><em>Harmonia axyridis</em> clone DS_3_034 18S ribosomal RNA gene, partial sequence</td>
<td>AAGCTTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
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<td>100</td>
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<tr>
<td>24</td>
<td><em>Biomphalaria glabrata</em> (chitinase–like protein PB1E7.04c, partial mRNA)</td>
<td>AAGCTTTTATTTAACCGCTCACACCACCTGACGTGTAAATAAGTTATTAGCCTGCCG GATCACGGCGTTACTCGTGCGTTAGATGTATTGATCTACATGTGGAGTCATCCATCAGGGCATCCAGGACCCCGAAGCAGCGGCCCTATCCGATCCGGTGCCTCCCTGGGGACCTCCCTCCCTGCTACTTGAGTAATGGGATTCAAAACACCACTTGGCGGCTGCTGTCGTGACAAAGACGATCCATTAAGGAGCGAGTCTTCCTCCTCGTACTTGACGTCGCCCATCGCTGCGGGCTAAATCCGAGCCGGGGGGCCGAACCCTTAGCACACCCCCTCCCCACATAACTTATTTCGACCGTATATATGGGGGGGAATATTGAAAATCCACTCCGATGGGGCTAGGATGGAGAGGGTTCGTTAGCCTGACCGAAGATGAGGTGTTGGGGGTAACAGCATCCATTGTCTCACGTGGGGGAACGTGATTATTTACGGGGTTGGCTGGTATGAATTC</td>
<td>523</td>
<td>100</td>
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*Note:* The above table includes sequences for various organisms and their respective gene functional analysis using Gateway Cloning System. Each sequence is listed along with its length and query coverage.
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.

After cloning *H. axyridis* ecdysone receptor A into a T–Blunt vector, plasmids were digested with *Eco*RI and *Hind*III 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 *Eco*RI and *Hind*III 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 *Hpa*I 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).
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 dsEcRA into 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-
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. Ecdysoid (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 (Tian and Palli, 2008). Furthermore, when dsBGEcR–A was injected into 6th–instar nymphs of *Blattella germanica*, most of the imagos 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–cultur- ing 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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