Construction of Henosepilachna Vigintioctomaculata cDNA Library and Identification of dsRNA Injection-Induced RNAi Effect

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Construction of *Henosepilachna Vigintioctomaculata* cDNA Library and Identification of dsRNA Injection–Induced RNAi Effect

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Henosepilachna vigintioctomaculata is a pest that damages plants of the Solanaceae family. To control of *H. vigintioctomaculata* through RNAi, which has received much attention in recent years, we investigated the effect of RNAi by injecting dsRNAs and constructing a cDNA library for selecting the genes to be targeted by RNAi. The N20 primer was used to synthesize cDNA of different sizes by removing the poly A. After amplification, PCR products of sizes varying between 200 and 600 bp were obtained. For the efficient cloning of the different genes, the products were cloned into a pDONR 207 vector using the Gateway system for constructing the cDNA library. The cDNA library thus constructed had a titer of 3.15×10^5 cfu/ml. The genes from the cDNA library were cloned into a LITMUS 28i vector containing the T7 promoter for synthesizing dsRNA. Electro–transformation was performed in the *E. coli* cells, and 48 colonies were randomly selected to confirm the size and duplication of the insert. The insert sizes varied between 100 and 500 bp and the different genes were cloned without duplicating the sequences. The results showed that 23 genes were those of insects, including those of the order Coleoptera, while the rest were genes from non–insect species. Using the T7 promoter of the LITMUS 28i vector, the genes with identified sequence and gene information were used for synthesizing 200–600 bp dsRNAs suitable for RNAi. Most of the larvae injected with *Hv1, Hv4*, and *Hv7* genes turned black and died within 7–10 days after injection.

Key words: *Henosepilachna vigintioctomaculata; Lycium chinese*; cDNA library; RNAi effect

INTRODUCTION

Henosepilachna vigintioctomaculata is an insect belonging to subfamily Epilachninae of the Coccinellidae family, under the order Coleoptera. It is distributed mainly in the northern part of central Korea. It also inhabits Japan, Taiwan and Eastern Siberia (Kwon *et al*., 2010). Since it is mainly distributed in Asia, most of the studies on *H. vigintioctomaculata* have been carried out in Japan and China, including studies on its host range and food preference, biological control using Bt toxin or natural parasitic enemies, effect of temperature conditions on its developmental characteristics, and the effect of using environment–friendly materials for its control, as well as on the molecular biological classification of Epilachninae (Hoshikawa, 1983; Kang *et al*., 2014; Kobayashi *et al*., 1998; Lee *et al*., 1988; Song *et al*., 2008).

Unlike *Harmonia axyridis*, which is generally carnivorous, *H. vigintioctomaculata* is a phytophagous insect, appearing especially in the fields adjacent to the mountains, three times a year, from June to July, from the end of July to the end of August, and in September (Hori *et al*., 2011). The larvae and imago damage plants of the Solanaceae family including *Lycium chinese*, potato, eggplant, black nightshade, and tomato.

At present, studies on pest control using RNA interference (RNAi) are being actively carried out. RNAi is a mechanism by which the target mRNA is degraded by the complementary dsRNA strand for inhibiting gene expression. In other words, when the dsRNA is introduced into the cell, it is cleaved into an siRNA having a size of 21–25 bp, by an enzyme called Dicer having RNase Ⅲ activity. The siRNA thus produced disassociates into single strands and binds to the RNA–induced silencing complex (RISC), and then to the complementary mRNA strand, thereby forming a dsRNA again. The dsRNA is cleaved by the Dicer protein, which suppresses the expression of the gene (Bell'es, 2010). In recent years, RNAi has emerged as a new alternative for the hygienic control of a variety of agricultural pests. Thus, studies have been conducted to increase the susceptibility of pests to Bt toxin or drugs through RNAi, or to achieve pest control by knocking down the major genes of insect pests (Bautista *et al*., 2009; Lycett *et al*., 2006; Pitino *et al*., 2011; Hu *et al*., 2016). Selecting the suitable target genes and the use of appropriate dsRNA delivery methods are important for an effective RNAi (Burand and Hunter, 2013).

In this study, we identified a number of genes and constructed a cDNA library for selecting the target genes suitable for RNAi to control *H. vigintioctomaculata*, as well as to investigate the function of the genes in *H. vigintioctomaculata* using RNAi. The cDNA library was constructed using the Gateway system. The Gateway system is a cloning method that uses the site–specific recombination method of bacteriophage λ. If the specific

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nucleotide sequence ATT is present, various genes can be easily cloned. Thus, this method is very efficient and has a high cloning accuracy because of the nature of the recombinase of bacteriophage λ, which has site–specific recombination properties (Hartley *et al*., 2000). In addition, we synthesized dsRNAs to efficiently transduce the genes from the prepared cDNA library into the insect. The genes were injected into the fourth larval stage of *H. vigintioctomaculata* and were screened for confirming the effect of RNAi.

MATERIALS AND METHODS

Test insect

Henosepilachna vigintioctomaculata was successively bred in an insect breeding room (insectarium) at 25 ± 1˚C and 50–60% relative humidity, under a 16L: 8D photoperiod. It was fed on a diet consisting of plants belonging to the Solanaceae family. *H. vigintioctomaculata* was successfully bred using the method proposed by Kang and coworkers (2014), with slight modifications. First, a Ø90 mm filter paper soaked in water, was placed on a cylindrical insect breeding dish (SPL, 100 × 40 mm), and the leaves of the solanaceous plants were placed on the filter paper. The first instar larvae of *H. vigintioctomaculata* were placed on top of this arrangement. Feed was replaced daily. The instar status of the larvae was determined according to the presence or absence of larval exuvium. The fourth instar larvae were used for dsRNA injection experiments.

Preparation of cDNA library

The cDNA library was constructed using the CloneMiner™Ⅱ cDNA library Construction Kit (Invitrogen, USA), (Fig. 1) and the methods of Ko and Youn (2015) and Jung (2016) as references.

Extraction of total RNA

Liquid nitrogen and the imagos of *H. vigintioctomaculata* were added to a mortar and finely ground. The samples were placed in 50 ml falcon tubes frozen in liquid nitrogen, to which 4 ml of Trizol reagent (MRC) was added, and the mixture was vortexed for dissolution. The dissolved samples were divided into eight 1.5 ml Eppendorf tubes in 500 μl aliquots. The same volume of chloroform was added to each sample, and the sample was vortexed for 1 min and then centrifuged at 15,000 rpm at 4˚C for 10 min. After centrifugation, the supernatant was transferred to a new 1.5 ml Eppendorf tube, and the 500 μl of isopropanol was added. After 20 inversions, the mixture was stored at 24 ± 2˚C for 10 min and then centrifuged at 15,000 rpm at 4˚C for 20 min to precipitate total RNA. After removing the supernatant, 1 ml of 80% ethanol was added, and the sample was centrifuged at 15,000 rpm at 4˚C for 10 min to wash total RNA. After centrifugation, the supernatant was discarded, and the remaining ethanol was completely removed. The total RNA pellet was dissolved in 300 μl of diethyl pyrocarbonate(DEPC)–treated water.

Total RNA was subsequently purified by the phenol/chloroform extraction method to increase its purity. Aqua phenol (300 μl) was added to each sample, and the sample was vortexed and centrifuged at 15,000 rpm at 4˚C for 15 min. After centrifugation, the supernatant was transferred to a new 1.5 ml Eppendorf tube, and the 500 μl of chloroform was added. Then, the sample was vortexed and centrifuged at 15,000 rpm at 4˚C for 10 min. The supernatant was transferred to a new 1.5 ml Eppendorf tube, precipitated with ethanol, and dissolved in 100 μl of DEPC–treated water.

mRNA isolation

The mRNA was isolated from 400 μl of total RNA (277 μg) using FastTrack® MAG mRNA Isolation Kits (Invitrogen) and stored at –80˚C through ethanol precipitation until the first strand of cDNA was synthesized.

Synthesis of the first cDNA strand

To prepare random primers for the first cDNA strand, the restriction site of the enzyme *Hin*dⅢ was added to the 3' end of the primer, as per instructions in the CloneMiner™ cDNA library Construction Kit. Instead of oligo (dTs), 20 Ns were added to induce random binding between the primers and the mRNAs (Table 1). A priming mixture was prepared by mixing 25.5 μl of mRNA (4.9 μg) and 2 μl of the first strand synthesis primer in a 1.5 ml Eppendorf tube. The mixture was incubated at 70˚C for 7 min and stored at room temperature for 10–15 min until the temperature of the mixture decreased to 45˚C (Table 1). While the temperature of the mixture was decreasing, 10 μl of 5X first–strand buffer, 5 μl of 0.1 M DTT, and 2.5 μl of 10 mM dNTPs were mixed in a new 1.5 ml Eppendorf tube to prepare the first strand mixture. The first strand mixture was then added to the priming mixture that had been cooled down to 45˚C, and the two mixtures were allowed to react at 45˚C for 2 min. Then, 2 μl of SuperScript®Ⅲ RT was added to the mixture and was mixed. The mixture was then transferred to a PCR tube and allowed to react at 45˚C for 20 min, at 50˚C for 20 min, and at 55˚C for 20 min, for synthesizing the first strand of the cDNA. The remaining mRNA was removed by treating with RNaseI (Ambion, USA), and the cDNA was dissolved in 23 μl of distilled water after ethanol precipitation.

5' attB1 adapter ligation

The 5' attB1 adapter was constructed by attaching the *Eco*RI sequence to the 5' end of the adapter, according to the protocol prescribed in the CloneMiner™ cDNA library Construction Kit (Table 1). The first strand of the cDNA (22 μl), 10 μl of 5X adapter buffer, 8 μl of 0.1 M DTT, 5 μl of T4 DNA ligase, and 5 μl of 5X adapter mix (10 pmol) were added to a 1.5 ml Eppendorf tube and mixed. The adapter was allowed to react at 16˚C for 20 h for ligation. After the reaction, ethanol precipitation was performed, and the adapter was dissolved in 81 μl of distilled water.

Synthesis of the second cDNA strand

The second strand of the cDNA was synthesized

using LA Taq™ DNA polymerase (Takara, Japan). The adapter–ligated cDNA (79 μl), 10 μl of 10X LA bufferII, 4 μl of 10 mM dNTP mix, v μl of LA Taq™ DNA polymerase (5 U/µl), 1 μl of the second strand cDNA synthesis primer (10 pmol), and 5 μl of $MgCl_2$ were added and mixed in a PCR tube. The mixture was then incubated at 68˚C for 20 min and at 72˚C for 20 min (Table 1). After the reaction, the mixture was dissolved in 20 μl of distilled water after ethanol precipitation.

PCR

PCR was performed using the LA Taq™ DNA polymerase (Takara, Japan). The 5' and 3' primers for PCR were constructed based on the sequences of the attB1 adapter U and the first strand synthesis primer, respectively (Table 1). In a PCR tube, 2 μl of cDNA, 2 μl of 10X LA buffer, 2 μl of dNTP mix, 1 μl of 5' primer (10 pmol), 1 μl of 3' primer (10 pmol), 1 μl of $MgCl_2$, 0.3 μl of LA Taq™ DNA polymerase, and 10.7 μl of distilled water were added and mixed. After an initial denaturation at 95˚C for 5 min, final denaturation occurred at

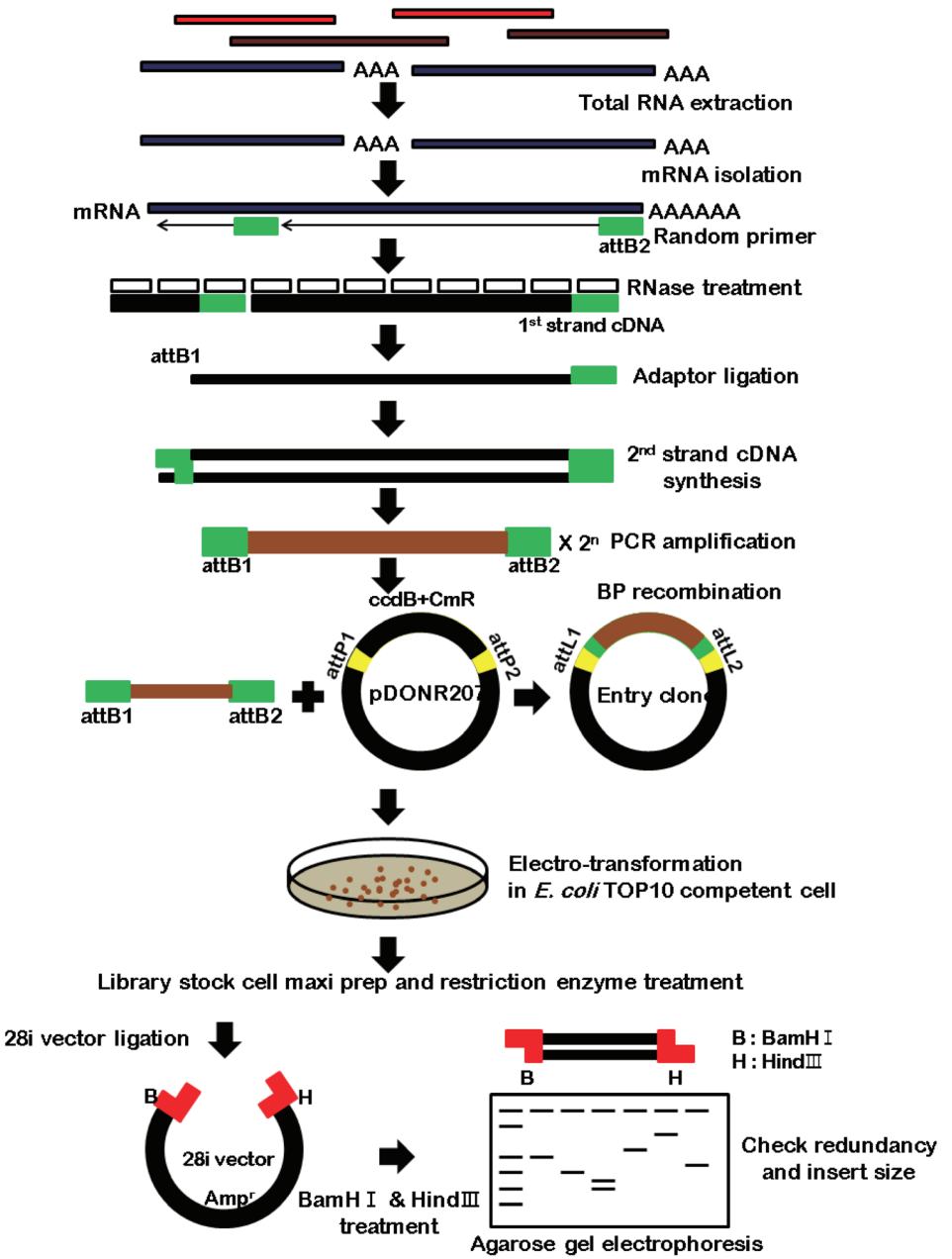

Fig. 1. Process of *Henosepilachna vigintioctomaculata* cDNA library construction. After mRNA isolation, the first strand of the cDNA was synthesized using a random primer by including the sequences of the restriction enzymes *Hin*dIII and *att*B2. The adapter, consisting of the sequences of the restriction enzymes *Eco*RI and *att*B1, was ligated to the 5' end of the first strand of the cDNA, for synthesizing the second cDNA strand. After PCR amplification, BP recombination was performed using the pDONR 207 vector and transformation in the TOP10 competent *E. coli* cells.

95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, for 35 cycles. Extension was then performed at 72°C for 5 min. The results of the PCR were confirmed by electrophoresis.

BP recombination

BP recombination was performed using the attP sequence of *H. vigintioctomaculata* cDNA and the attP sequence of the pDONR 207 vector (Fig. 2). In a 1.5 ml Eppendorf tube, 3 μl of cDNA, 4 μl of pDONR vector (1 μl/μg), and 3 μl of the BP clonase®Ⅱ enzyme mix were added, mixed, and made to react at 25°C for 20 h. Two microliters of proteinase K was added to stop the BP recombination, followed by BP recombination stopping reaction at 37°C for 15 min and at 75°C for 10 min. After the reaction, ethanol precipitation was performed, and the mixture was finally dissolved in 10 μl of distilled water.

Electro–transformation

The BP reaction sample (1.5 μl) was applied to TOP10 Electrocompetent™ *E. coli* cells (Invitrogen, USA) and tapped. The cells were then subjected to electric shock from an electroporator using a cuvette. During this time, the electroporator was set at 1.8 kV, 200 Ω, and 25 μF. Afterwards, 1 ml of S.O.C medium was added to the cuvette, transferred to a new 1.5 ml Eppendorf tube, and cultured by placing in a shaker inside an incubator at 37°C for 1 h. After incubation, 900 μl of the S.O.C. medium was aliquoted into three 300 μl units and mixed with the same volume of 40% glycerol in the ratio 1:1. The prepared stock cells were stored at 80°C, and the remaining 100 μl of the S.O.C. medium was diluted to 10^{-1}–10^{-4} folds using LB broth. After this, 100 μl of the diluted medium was streaked on a medium containing gentamycin and cultured at 37°C for 18 h. After culturing, the number of colonies was counted for measuring the titer of the cDNA library.

LITMUS 28i vector cloning

PCR of the cDNA library

The constructed cDNA library was subjected to PCR for cloning the inserts of the cDNA library into the LITMUS 28i vector (NEB, USA). First, the cDNA library stock cells were gently scraped for use as a PCR template and cultured in 2 ml of LB broth containing gentamycin. The plasmids were extracted with the alkaline lysis method (Birnboim and Doly, 1979) and dissolved in 500 μl of distilled water.

The PCR mixture consisted of the following: 1 μl of DNA template, 2 μl of 10X buffer, 2 μl of dNTPs, 1 μl of 5' primer (10 pmol), 1 μl of 3' primer (10 pmol), 0.3 μl of Prime Taq DNA polymerase, and 12.7 μl of distilled water. The initial denaturation was performed at 95°C for 5 min, final denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, for 35

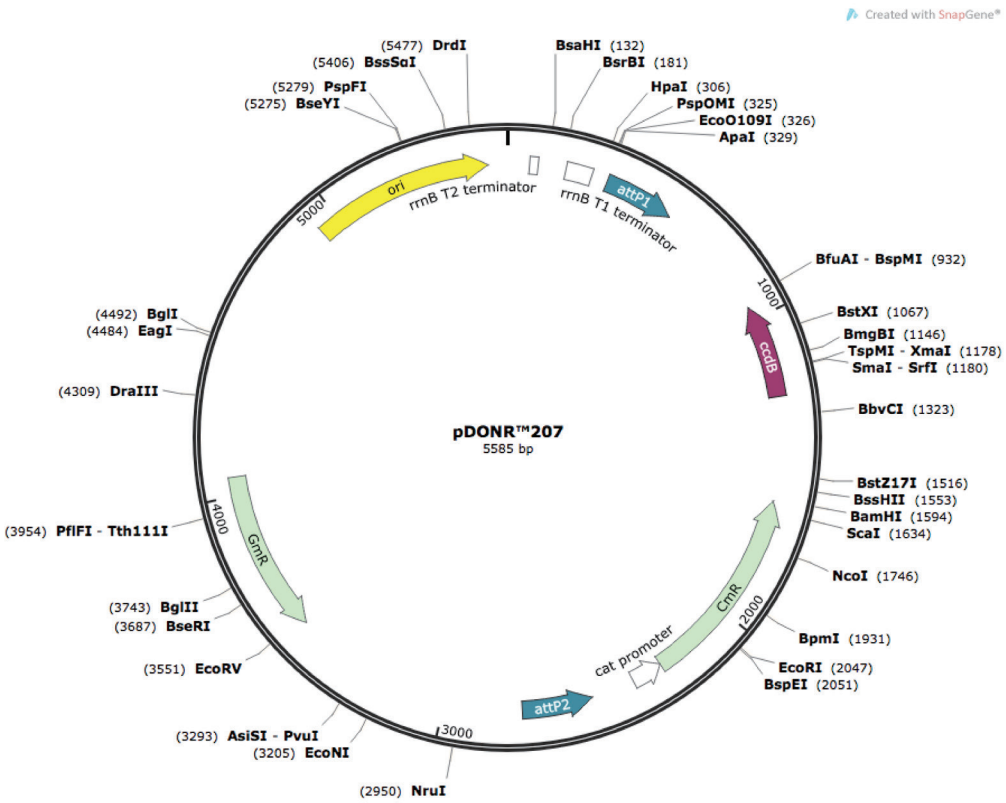

Fig. 2. pDONR™207 vector (Invitrogen, USA) map. The attP site is a specific recombination site in bacteriophage λ that allows cloning by recombination from attB flanked DNAs or gateway expression clones. The ccdB gene and the chloramphenicol resistance gene are used to select the negative plasmid (original vector).

cycles. Then, the final extension was performed at 72°C for 5 min (Table 1). To increase the concentration, PCR was performed 10 times. The PCR products were collected, purified using a PCR purification kit (NucleoGen, Korea), and dissolved in 100 μl of distilled water.

Treatment with the restriction enzymes EcoRI and HindIII

The PCR products and the LITMUS 28i transcription vector were treated with *Eco*RI and *Hin*dIII (NEB, USA) for cloning the PCR products into the LITMUS 28i transcription vector. The composition of the restriction enzyme mixture was 7 μl of either the PCR products or the vector, 0.5 μl of *Eco*RI, 0.5 μl of *Hin*dIII, 1 μl of 2.1 NEB buffer, and 1 μl of distilled water. The mixture was allowed to react at 37°C for 4 h.

Ligation

The restriction enzyme–treated insert and the LITMUS 28i transcription vector were ligated using the T4 DNA ligase (Takara, Japan) (Fig. 3). The ligation mixture comprised of 6.5 μl of insert DNA, 1.5 μl of vector, 1 μl of 10X buffer, and 1 μl of T4 DNA ligase. The mixture was allowed to react at room temperature for 16 h. After the reaction, the mixture was stored at –20°C.

Electro–transformation

The ligated samples were transformed into ElectroMAX™ DH10B *E. coli* cells (Invitrogen, USA) via an electroporator. During this time, the electroporator was set to 1.8 kV, 200 Ω, and 25 μF. The transformed samples were mixed with 1 ml of S.O.C medium and cultured by placing in a shaker within an incubator at 37°C for 1 h. The cultured stock solution (900 μl) was aliquoted into three 300 μl units and mixed with the same volume of 40% glycerol in the ratio 1:1. The prepared stock cells were stored at –80°C. The remaining 100 μl of the cultured stock solution was diluted to 10^{-1}–10^{-4} folds using LB broth, and 100 μl of the diluted solution was streaked in the medium containing the antibiotic ampicillin and cultured overnight at 37°C.

Analysis of the insert size and duplication

The size and duplication of the inserts of the *H. vigintioctomaculata* cDNA library were determined using the restriction enzymes *Eco*RI and *Hin*dIII. Following electro–transformation, the cells were first streaked on an ampicillin medium. The 48 colonies identified were randomly selected, and were individually cultured in 3 ml of LB broth containing ampicillin. The plasmid DNA was subsequently extracted by the alkaline lysis method. For treatment with the restriction enzymes, 7 μl of plasmid DNA, 0.5 μl of EcoRI, 0.5 μl of *Hin*dIII, 1 μl of 2.1 NEB buffer, and 1 μl of distilled water were added to the PCR tube and mixed. The mixture was then made to react at 37°C for 4 h. The results of restriction enzyme digestion were confirmed by electrophoresis. Information pertaining to the inserts was retrieved by NCBI BLAST search, which provided the sequence information obtained from the sequencing experiments.

In vitro transcription

Treatment with HpaI

The plasmid DNA was treated with the restriction enzyme *Hpa*I (NEB, USA) to construct a linear DNA template for dsRNA synthesis. The *E. coli* cells contain-

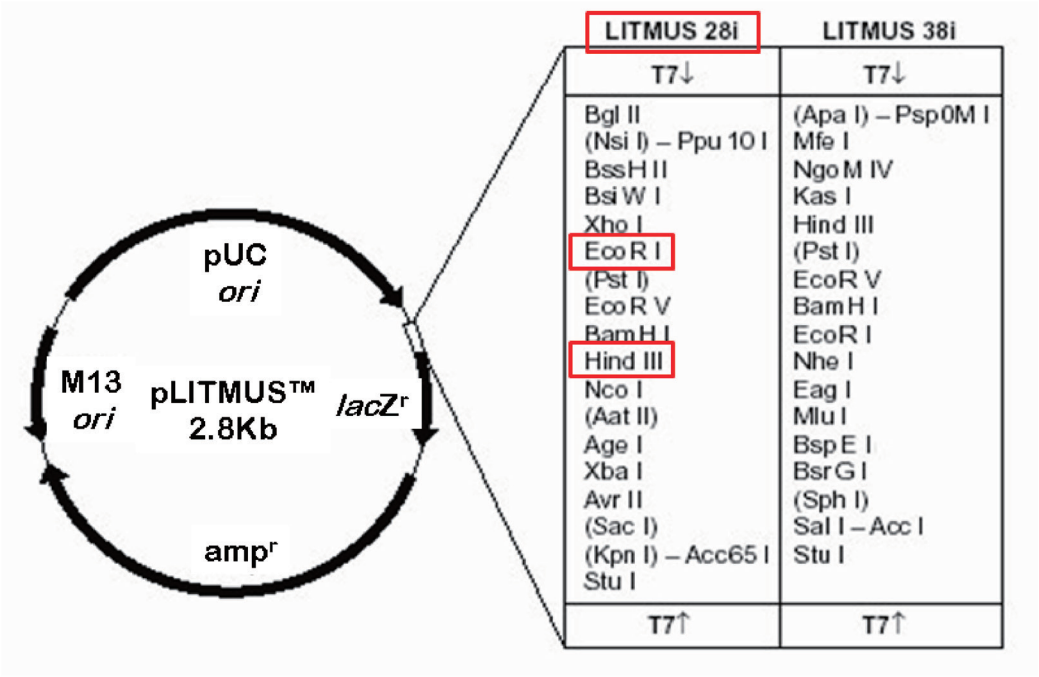

Fig. 3. LITMUS 28i vector (NEB, UK) map. The selected genes were inserted into the LITMUS 28i vector using the restriction enzymes *Eco*RI and *Hin*dIII. The ampicillin resistance gene was used to select the LITMUS 28i vector. We synthesized the dsRNA of the selected gene using T7 promoters.

ing the individual genes from the constructed cDNA library were cultured in 3 ml of LB broth containing the antibiotic ampicillin, and the plasmid DNA was extracted with the alkaline lysis method.

For treatment with the restriction enzymes, 7 μl of plasmid DNA, 1 μl of CutSmart, 1 μl of *Hpa*I, and 1 μl of distilled water were added to the PCR tube and mixed well, followed by reaction at 37°C for 4 h. To increase the concentration, the treatment with the restriction enzymes was repeated five times for each gene. After the reaction, the cells were collected in a 1.5 ml Eppendorf tube and dissolved in 20 μl of distilled water after ethanol precipitation.

Synthesis of dsRNA

The dsRNA of the genes from the cDNA library were synthesized using the T7 RiboMAX™ Express RNAi System kit (Promega). First, 10 μl of 2X buffer, 2 μl of the enzyme mix, and 6 μl of nuclease–free water were added to 2 μl of linear DNA template, and allowed to react at 37°C for 2 h to synthesize the two strands of RNA. The RNA strands were incubated at 70°C for 10 min and at room temperature for 20 min for allowing base pairing between the complementary strands of RNA. Then, 2 μl of DNase, which had been diluted 200–fold with distilled water, was mixed with 2 μl of RNase, which had been diluted 200–fold with DEPC–treated water, and the two were allowed to react at 37°C for 30 min. After ethanol precipitation, the sample was dissolved in distilled water until the concentration was 1 μg/µl (Fig. 4).

Injecting dsRNA into H. vigintioctomaculata larvae

To screen for the 48 genes previously identified, the synthesized dsRNA was injected into the fourth instar larvae of *H. vigintioctomaculata*. Ten fourth instar larvae of *H. vigintioctomaculata* were used in each treatment group. Distilled water and 1 μl of dsRNA (1 μg/μl) were injected into the first and second abdominal segments of each larvae in the untreated (control) and treated groups, respectively, using a nano injector (Drummond, USA) (Niimi *et al*., 2005). The larvae of *H. vigintioctomaculata* used in the experiments were fed with solanaceous plants in the insectarium, and the effect of RNAi on their phenotypic variation, mortality, and normal emergence were studied.

RESULTS

Construction of a cDNA library

PCR amplification

To amplify the cDNA containing the attB sequence, PCR was performed using the first strand and second strand synthesis primers (Table 1), and electrophoresis was performed to confirm the amplification of cDNAs of different sizes. The result of PCR demonstrated a band corresponding to 200 to 600 bp (Fig. 5), thereby confirming that cDNA of sizes varying between 100 and 500 bp were amplified, leaving out the adapters at both ends.

Titer of the cDNA library

The cDNA with attB adapters at both ends underwent BP recombination with the pDONR 207™ vector

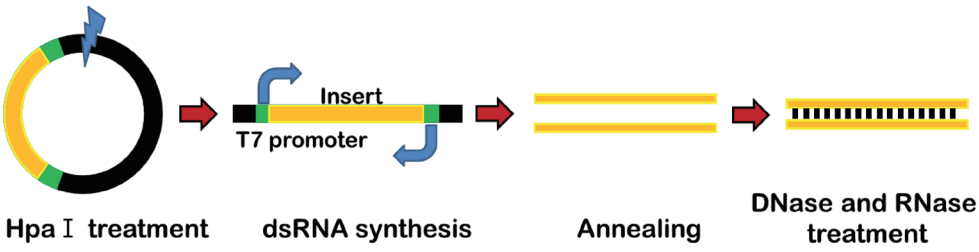

Fig. 4. *In vitro* transcription process. To construct linear DNA, circular DNA was treated with the restriction enzyme *Hpa*I. The RNA strands were individually synthesized using the T7 promoter of the LITMUS 28i vector. The two complementary strands of RNA anneal as the reaction temperature decreases. Finally, the solution was treated with DNase and RNase to remove the DNA and ssRNA from the solution.

Table 1. Primers used for the construction of the cDNA library. Bold letters indicate restriction enzymes

Primers		Sequences (5'–3')
First strand synthesis		Biotin–GGGG ACA ACT TTG TAC AAG AAA GTT GGG **AAG CTT** N_{20}
attB1 adapter (Double strand)	U	TCG TCG GGG ACA ACT TTG TAC AAA AAA GTT GG**G AAT TC**
	L	P^{1} **GAA TTC** CCA ACT TTT TTG TAC AAA GTT GTC CCC
Second strand synthesis		TCG TCG GGG ACA ACT TTG TAC AAA AAA GTT GG**G AAT TC**
PCR	5'	TCG TCG GGG ACA ACT TTG TAC AAA AAA GTT GG**G AAT TC**
	3'	GGGG ACA ACT TTG TAC AAG AAA GTT GGG **AAG CTT**

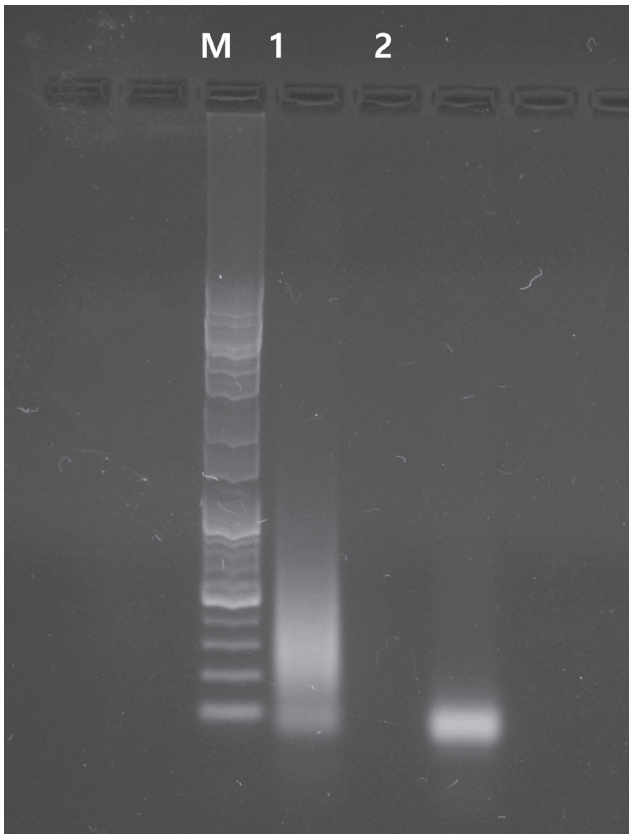

Fig. 5. Electrophoresis of *Henosepilachna vigintioctomaculata* cDNA amplified using 5' and 3' primers. M: 1 kb plus ladder marker; Lane 1: PCR product; Lane 2: Negative control.

(Invitrogen, USA) and were then transformed into Electrocompetent™ TOP10 *E. coli* cells (Invitrogen, USA). The culture solution was continually diluted until the final concentration reached 10^{-4} fold, and 100 μl of the resulting solution was plated on an LB plate medium containing gentamycin. The cells were subsequently cultured. Following culture, the number of colonies growing on the medium was calculated to determine the titer of the cDNA library. After counting, 556 colonies were found to grow in the medium diluted 10^{-2} fold and had been streaked with the solution diluted 10^{-1} fold; 42 colonies were found in the medium diluted 10^{-3} fold and had been streaked with the solution diluted 10^{-2} fold; and 6 colonies were found in the medium diluted 10^{-4} fold and had been streaked with the solution diluted 10^{-3} fold. The final titer of the cDNA library was 3.15×10^5 cfu/ml based on the number of colonies (Table 2).

LITMUS 28i vector cloning: Identification of inserts

Identification of insert size

The constructed cDNA library was cloned into a LITMUS 28i vector using restriction enzymes *Eco*RI and *Hin*dIII to synthesize dsRNAs for injecting into the larvae of *H. vigintioctomaculata*. After cloning, 48 colonies were randomly selected to confirm the size of the inserts, and were digested with the restriction enzymes *Eco*RI and *Hin*dIII, followed by electrophoresis. As a result, an insert band of 100 to 500 bp was observed in addition to the band corresponding to the LITMUS 28i vector, which was nearly 3 kb (Fig. 6).

Sequencing and NCBI BLAST search

To identify the gene information pertaining to the constructed cDNA library, the 48 plasmids with confirmed inserts were first sequenced, and NCBI BLAST search was performed based on the nucleotide sequences obtained. The results demonstrated that none of the genes had been duplicated. The DNA BLAST search (Table 3) and the EST blast search (Table 4) identified 23 and 19 insect genes (Table 5), respectively, out of the 48 genes.

Random *in vitro* gene transcription

HpaI treatment

The LITMUS 28i vector containing the genes from the cDNA library was treated with *Hpa*Ito convert the circular DNA into a linear form prior to synthesis of the dsRNA, and linearization was confirmed by electrophoresis (Fig. 7).

Synthesis of dsRNAs of random genes

The dsRNA of random genes from the cDNA library were synthesized using the linear DNA identified in Fig. 6 as templates. The dsRNA band corresponding to a size of 200–600 bp was confirmed by electrophoresis (Fig. 8).

Effects of dsRNA injection on larval mortality

Fifteen out of 26 dsRNA synthesized from the genes in the cDNA library were injected into the abdomen of the fourth instar larvae of *H. vigintioctomaculata*. Most of the larvae injected with distilled water in the control setup underwent normal emergence. In contrast, most of the larvae treated with *Hv1*, *Hv4*, and *Hv7* genes did not metamorphose into pupae, and instead turned black and died within 7–10 days after injection (Fig. 9 and Table 6).

Table 2. *Henosepilachna vigintioctomaculata* cDNA library titer

Dilution	Amount plated (μl)	Colonies per plate	Titer (cfu/ml)	Average titer (cfu/ml)	Total volume (ml)	Total CFUs (cfu)
10^{-2}	100	556	5.56×10^4			
10^{-3}	100	42	4.2×10^4	4.54×10^4	6	3.15×10^5
10^{-4}	100	6	6.0×10^4			

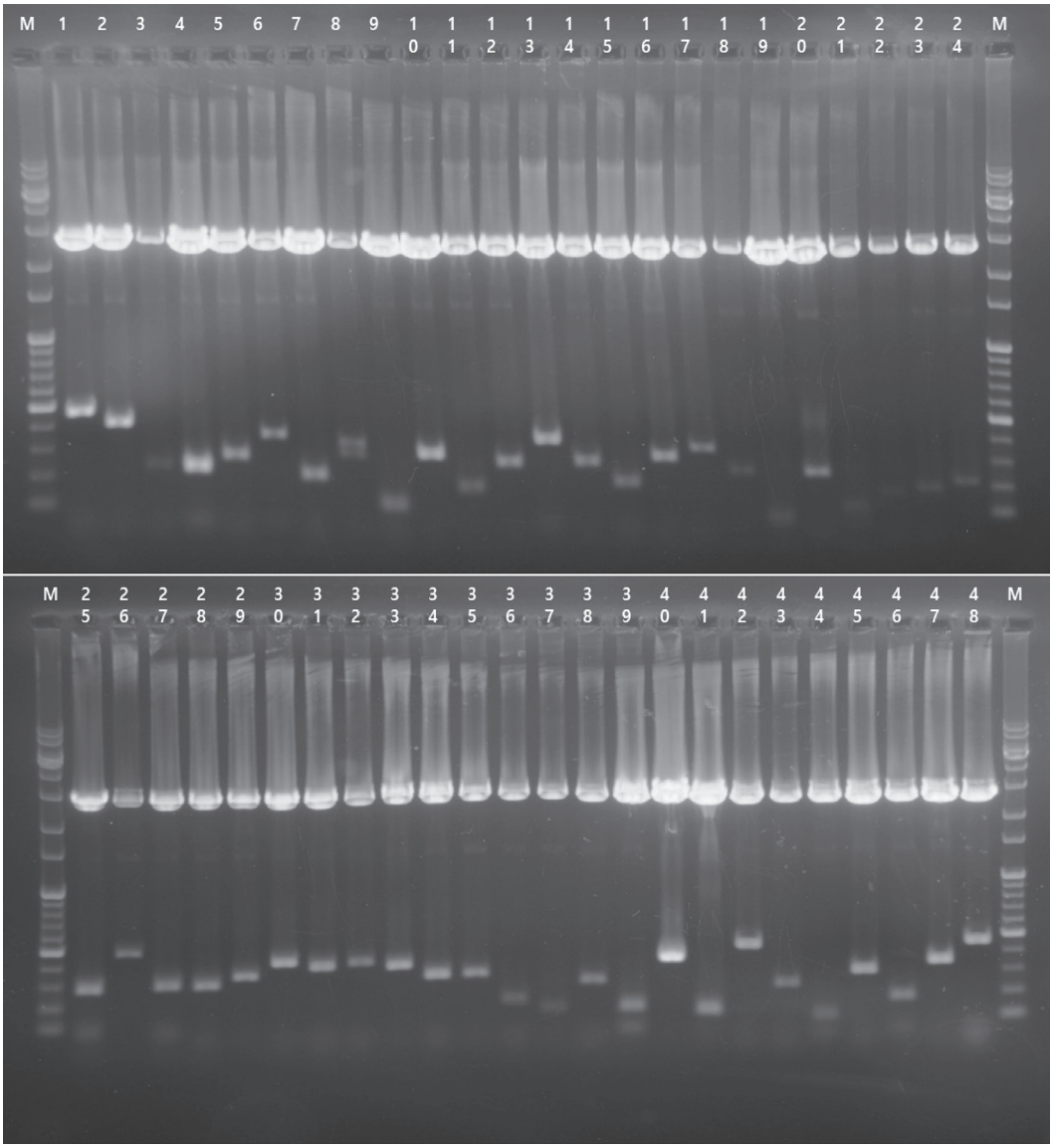

Fig. 6. Electrophoresis of the inserts from the cDNA library after cloning into the LITMUS 28i transcription vector. Plasmids from 48 randomly selected colonies were electrophoresed on an agarose gel and analyzed to confirm the size of the inserts using restriction enzymes *Eco*RI and *Hin*dIII. Excluding the size corresponding to the vector (2.8 kb), the size of the cDNA inserts was confirmed to be within 100 to 500 bp. M: 1 kb plus DNA ladder; Lanes 1 to 48: cDNA library inserts.

Table 3. Results of the DNA BLAST search of the *Henosepilachna vigintioctomaculata* cDNA library using 48 randomly–selected colonies for checking redundancy

	DNA blast search	Size (bp)	Accession	Identity (%)
No. 1	PREDICTED: *Alligator sinensis* cytochrome P450 2K4–like (LOC102368184), mRNA	475	XM_006039181.2	79
No. 2	PREDICTED: *Anoplophora glabripennis* serine/threonine–protein kinase MARK2–like (LOC108917417), transcript variant X10, mRNA.	414	XM_018723989.1	79
No. 3	*Cyprinus carpio* genome assembly common carp genome, scaffold 000001188	232	LN_591414.1	84
No. 4	*Trypanosoma cruzi cruzi* strain Sylvio X10/cl1 chromosome TcI9 sequence	234	CP_015659.1	85
No. 5	PREDICTED: *Tribolium castaneum* troponin T, skeletal muscle (LOC663893), transcript variant X7, mRNA	282	XM_008203244.2	82

	DNA blast search	Size (bp)	Accession	Identity (%)
No. 6	PREDICTED: *Anoplophora glabripennis* U1 small nuclear ribonucleoprotein 70 kDa (LOC108915274), mRNA	370	XM_018721256.1	71
No. 7	PREDICTED: *Anoplophora glabripennis* cell growth regulator with RING finger domain protein 1–like (LOC108917274), mRNA.	203	XM_018723783.1	80
No. 8	PREDICTED: *Anoplophora glabripennis* pleckstrin homology–like domain family B member 2 (LOC108912609), mRNA.	299	XM_018717887.1	68
No. 9	*Salmo salar* clone Contig1229 40S ribosomal protein S20 putative mRNA, complete cds	109	BT_058173.1	100
No. 10	PREDICTED: *Tribolium castaneum* pupal cuticle protein 36 (LOC100141541), transcript variant X5, mRNA	286	XM_008194105.2	73
No. 11	*Medicago truncatula* clone mth2–7k2, complete sequence	177	AC_144340.30	85
No. 12	PREDICTED: *Cicer arietinum* chromodomain–helicase–DNA–binding protein 3–like (LOC101506720), transcript variant X10, mRNA	266	XM_012713963.1	91
No. 13	PREDICTED: *Amyelois transitella* platelet–activating factor acetylhydrolase IB subunit beta homolog (LOC106129028), mRNA	372	XM_013327461.1	82
No. 14	PREDICTED: *Tribolium castaneum* death–inducer obliterator 1 (LOC658430), transcript variant X2, mRNA	275	XM_964818.4	71
No. 15	*Dendroctonus ponderosae* Seq01036971, whole genome shotgun sequence	190	APGK_01036961.1	77
No. 16	PREDICTED: *Tribolium castaneum* eukaryotic translation initiation factor 5A (LOC663814), mRNA	292	XM_969849.3	80
No. 17	PREDICTED: *Tribolium castaneum* collagen alpha–2(IV) chain (LOC103313111), mRNA	339	XM_008195512.2	70
No. 18	PREDICTED: *Pyrus x bretschneideri* allantoate deiminase–like (LOC103932417), transcript variant X2, mRNA	250	XM_009342015.1	79
No. 19	PREDICTED: *Drosophila kikkawai* tubulin alpha–1 chain (LOC108081135), mRNA	74	XM_017176156.1	93
No. 20	*Heliobacterium modesticaldum* Ice1 strain Ice1, complete genome	246	CP_000930.2	81
No. 21	PREDICTED: *Cynoglossus semilaevis* desmoglein–2–like (LOC103399843), mRNA	118	XM_017031054.1	76
No. 22	PREDICTED: *Diaphorina citri* actin, muscle (LOC103506086), transcript variant X2, mRNA	177	XM_008470468.2	81
No. 23	PREDICTED: *Cephus cinctus* uncharacterized LOC107269745 (LOC107269745), transcript variant X2, mRNA	192	XM_015743953.1	79
No. 24	PREDICTED: *Athalia rosae* atrophin–1–like (LOC105688664), transcript variant X2, mRNA	216	XM_012405185.1	75
No. 25	*Mesocestoides corti* genome assembly M_corti_Specht_Voge, scaffold MCOS_contig0001148	283	LM_532355.1	100
No. 26	PREDICTED: *Anoplophora glabripennis* eukaryotic translation initiation factor 4E–binding protein Mextli (LOC108913008), transcript variant X3, mRNA	464	XM_018718463.1	73
No. 27	*Ovis canadensis* canadensis isolate 43U chromosome 5 sequence	292	CP_011890.1	89
No. 28	*Desulfovibrio magneticus* RS–1 DNA, complete genome	286	AP_010904.1	85
No. 29	PREDICTED: *Tribolium castaneum* serine/arginine–rich splicing factor 7 (LOC657225), transcript variant X3, mRNA	323	XM_015983532.1	78
No. 30	*Harmonia axyridis* holotricin–like peptide mRNA, partial cds	405	FJ_597735.1	97
No. 31	*Kluyveromyces lactis* NRRL Y–1140 hypothetical protein partial mRNA	374	XM_451674.1	72
No. 32	PREDICTED: *Crassostrea gigas* fibroblast growth factor receptor–like 1 (LOC105328717), transcript variant X4, mRNA	389	XM_011429712.1	97
No. 33	*Leptinotarsa decemlineata* putative juvenile hormone epoxide hydrolase 2 mRNA, complete cds	356	KP_271046.1	70

	DNA blast search	Size (bp)	Accession	Identity (%)
No. 34	*Schistosoma mattheei* genome assembly S_mattheei_Denwood, scaffold SMTD_scaffold0002181	301	LM_151531.1	100
No. 35	*Thiocystis violascens* DSM 198, complete genome	301	CP_003154.1	67
No. 36	*Synechococcus* phage ACG–2014g isolate Syn7803US105, complete genome	189	KJ_019071.1	75
No. 37	*Harmonia axyridis* elongation factor 1–alpha gene, partial cds	153	KP_677714.1	98
No. 38	*Apteryx australis* mantelli genome assembly AptMant0, scaffold scaffold32	275	LK_064646.1	84
No. 39	*Plasmodium cynomolgi* clone Pc675.1–5C merozoite surface protein 3 (MSP3B1) gene, partial cds	160	KC_907437.1	75
No. 40	*Nippostrongylus brasiliensis* genome assembly N_brasiliensis_RM07_v1_5_4, scaffold NBR_contig0003768	383	LM_446062.1	70
No. 41	*Vigna angularis* var. *angularis* DNA, chromosome 2, almost complete sequence, cultivar: Shumari	149	AP_015035.1	83
No. 42	PREDICTED: *Ficedula albicollis* senataxin (SETX), transcript variant X3, mRNA	475	XM_005055587.2	96
No. 43	*Schistosoma rodhaini* genome assembly S_rodhaini_Burundi, scaffold SROB_scaffold0001066	263	LL_958044.1	81
No. 44	PREDICTED: *Tribolium castaneum* probable ubiquitin carboxyl–terminal hydrolase FAF–X (LOC661487), transcript variant X3, mRNA	122	XM_008202251.2	77
No. 45	PREDICTED: *Tribolium castaneum* uncharacterized LOC661069 (LOC661069), mRNA	318	XM_967257.3	69
No. 46	PREDICTED: *Hyalella azteca* hornerin–like (LOC108681882), mRNA	190	XM_018170958.1	78
No. 47	PREDICTED: *Drosophila rhopaloa* mucin–2 (LOC108040627), mRNA	354	XM_017118179.1	72
No. 48	PREDICTED: *Sinocyclocheilus anshuiensis* collagen alpha–1(V) chain-like (LOC107675758), transcript variant X2, mRNA	464	XM_016470190.1	72

Table 4. EST blast search data of *Henosepilachna vigintioctomaculat*a cDNA library randomly–selected 48 colonies for checking redundancy

	EST blast search	Size (bp)	Accession	Identity (%)
No. 1	AGENCOURT_10504586 NIH_MGC_169 *Mus musculus* cDNA clone IMAGE:6707770 5', mRNA sequence	475	BU939486.1	78
No. 2	TO1003C05SP6 TO1 *Tribolium castaneum* cDNA, mRNA sequence	414	ES553718.1	77
No. 3	MR2–SN0005–220500–007–c08 SN0005 *Homo sapiens* cDNA, mRNA sequence	232	BE840857.1	93
No. 4	CCPN3461.b1 CCPN *Wrightia tinctoria* developing embryos (L) *Wrightia tinctoria* cDNA clone CCPN3461 5', mRNA sequence	234	HS560293.1	86
No. 5	ST020033B20F05 Normalized and subtracted western corn rootworm female head cDNA library *Diabrotica virgifera virgifera* cDNA clone ST020033B20F05 5', mRNA sequence	282	EW773496.1	81
No. 6	DPO1413.CR_L13 Dpo14-Larvae–Whole–Coldadapting–Norm (DPO14) *Dendroctonus ponderosae* cDNA clone DPO1413_L13 3', mRNA sequence	370	GT485650.1	72
No. 7	HTAB–aad00d04.b1 *Heterorhabditis_bacteriophora*_HTAB2_EST *Heterorhabditis bacteriophora* cDNA, mRNA sequence	203	EX010930.1	95
No. 8	FQ881770 *Sitophilus oryzae* normalized cDNA library *Sitophilus oryzae* cDNA, mRNA sequence	299	FQ881770.1	75
No. 9	FP460032 *Tuber melanosporum* fruit body *Tuber melanosporum* cDNA clone SY0AAC41YJ15 5', mRNA sequence	109	FP460032.1	100
No. 10	125689747 TH1 *Tribolium castaneum* cDNA clone 21C9 3', mRNA sequence	286	DT777540.1	74

	DNA blast search	Size (bp)	Accession	Identity (%)
No. 11	BW638886 planarian cDNA library *Dugesia ryukyuensis* cDNA clone Dr_sW_013_A05 5', mRNA sequence	177	BW638886.1	85
No. 12	EPP167KIAA11S002525 *Perilla frutescens* var. *frutescens* normalized full length cDNA library *Perilla frutescens* var. *frutescens* cDNA 5', mRNA sequence	266	JZ580805.1	85
No. 13	601566944F1 NIH_MGC_21 *Homo sapiens* cDNA clone IMAGE:3841594 5', mRNA sequence	372	BE732336.1	91
No. 14	USDA-FP_188778 *Lysiphlebus testaceipes* adult whole body *Lysiphlebus testaceipes* cDNA clone WLt091_B11 5', mRNA sequence	275	EH016261.1	77
No. 15	EST_omor_rgc_3804 omorrgc mixed_tissue *Osmerus mordax* cDNA *Osmerus mordax* cDNA clone omor_rgc_504_024_rev 5', mRNA sequence	190	EL539851.1	100
No. 16	HISTERID4POLYTR1_F02_1_047 Hister sp. cDNA *Hister sp*. APV–2005 cDNA clone HISTERID4POLYTR1_F02_1_047 3', mRNA sequence	292	CV159008.1	83
No. 17	HB0848 HB, healthy alfalfa leafcutting bee library *Megachile rotundata* cDNA clone HB0848 5' similar to collagen IV alpha 1 chain precursor, mRNA sequence	339	GD241671.1	67
No. 18	15266160 CERES–502 *Zea mays* cDNA clone 1513277 5', mRNA sequence	250	FL192392.1	91
No. 19	FQ836636 *Asobara tabida* normalized cDNA library *Asobara tabida* cDNA, mRNA sequence	74	FQ836636.1	95
No. 20	CLJ311–H12.y1d–s SHGC-CLJ2 *Gasterosteus aculeatus* cDNA clone CLJ311–H12 5', mRNA sequence	246	DW619661.1	74
No. 21	CAII3617.fwd CAII *Postia placenta* in rich medium *Postia placenta* cDNA clone CAII3617 5', mRNA sequence	118	FL620955.1	93
No. 22	BW638886 planarian cDNA library *Dugesia ryukyuensis* cDNA clone Dr_sW_013_A05 5', mRNA sequence	177	BW638886.1	85
No. 23	OtP020F06_021607f *Onthophagus taurus* pupal library *Onthophagus taurus* cDNA clone OtP020F06 5', mRNA sequence	192	FG542466.1	82
No. 24	127466896 TH1 *Tribolium castaneum* cDNA clone 125K23 5', mRNA sequence	216	DT784846.1	77
No. 25	S983494 *Arachis hypogaea* seedling *Arachis hypogaea* cDNA clone AHCS10M19, mRNA sequence	283	FS983494.1	77
No. 26	IPG002D10_249348 JH Ⅲ–treated male *I. pini* midguts *Ips pini* cDNA clone IPG002D10 5, mRNA sequence	464	CB408223.1	72
No. 27	UNQ89–P23.x1d–t UNQ (*Panicum virgatum* cv. Alamo–AP13) *Panicum virgatum* cDNA 5', mRNA sequence	292	JG872956.1	83
No. 28	CCOU3129.b1 CCOU *Avena barbata* leaf, pooled from different levels of rain and nitrogen (H) *Avena barbata* cDNA clone CCOU3129 5', mRNA sequence	286	GR336807.1	88
No. 29	FN640040 bom001no *Bombus terrestris* cDNA clone bom001noP0124M04 5', mRNA sequence	323	FN640040.1	77
No. 30	EST 11656 Guarana fruits cDNA library *Paullinia cupana* var. *sorbilis* cDNA, mRNA sequence	405	EC775160.1	67
No. 31	CCPO2650.b1 CCPO *Wrightia tinctoria* developing embryos (H) *Wrightia tinctoria* cDNA clone CCPO2650 5', mRNA sequence	374	HS567126.1	75
No. 32	FQ669515 *Crassostrea gigas* library (Genoscope – CEA) *Crassostrea gigas* cDNA clone WY0AAA60YD13FM1, mRNA sequence	389	FQ669515.1	97
No. 33	OtL011A08_010407c *Onthophagus taurus* larval library *Onthophagus taurus* cDNA clone OtL011A08 5', mRNA sequence	356	FG539876.1	72
No. 34	603498303F1 CSEQCHN65 *Gallus gallus* cDNA clone ChEST411f2 5', mRNA sequence	301	BU330346.1	87
No. 35	ZooX20012J11.g_040 *Symbiodinium* normalized stress library Lambda ZAP Ⅱ *Symbiodinium sp*. C3 cDNA 5', mRNA sequence	301	FE865205.1	94
No. 36	Physarum05692 *Physarum polycephalum* starvation stress library *Physarum polycephalum* cDNA clone PpolyN1a02f03.m13r, mRNA sequence	189	EL578212.1	81
No. 37	aar0afa6ym22cm1.1 *Leptopilina heterotoma* venom apparatus cDNA library *Leptopilina heterotoma* cDNA, mRNA sequence	153	JZ367432.1	88

	DNA blast search	Size (bp)	Accession	Identity (%)
No. 38	CCHT22304.b1_O07.ab1 CCHT Niger Seed *Guizotia abyssinica* cDNA clone CCHT22304, mRNA sequence	275	GE564407.1	88
No. 39	AM053282 *Epidinium ecaudatum* library (McEwan NR) *Epidinium ecaudatum* cDNA clone Eec05_C03, mRNA sequence	160	AM053282.1	78
No. 40	LB011116.CR_N22 GC_BGC–11 *Bos taurus* cDNA clone IMAGE:7343160 5', mRNA sequence	383	EH373754.1	82
No. 41	HX728364 full–length enriched *Ophiorrhiza pumila* cDNA library *Ophiorrhiza pumila* cDNA clone OPN019_M02, mRNA sequence	149	HX728364.1	94
No. 42	AGENCOURT_10504586 NIH_MGC_169 *Mus musculus* cDNA clone IMAGE:6707770 5', mRNA sequence	475	BU939486.1	78
No. 43	1108793333840 New World Screwworm Egg 9261 ESTs *Cochliomyia hominivorax* cDNA, mRNA sequence	263	FG291182.1	81
No. 44	ST020036B20C03 Normalized and subtracted western corn rootworm female head cDNA library *Diabrotica virgifera virgifera* cDNA clone ST020036B20C03 5', mRNA sequence	122	EW773942.1	74
No. 45	CmaE1_37_C01_T3 Cowpea weevil larvae Lambda Zap Express Library *Callosobruchus maculatus* cDNA, mRNA sequence	318	CB377412.1	70
No. 46	CNB182–A11.y1d–s SHGC–CNB *Gasterosteus aculeatus* cDNA clone CNB182–A11 5', mRNA sequence	190	DT996027.1	81
No. 47	BL–5895 *Nilaparvata lugens* illumina library *Nilaparvata lugens* cDNA 5', mRNA sequence	354	HS423729.1	77
No. 48	LE_MX1_51F10_SP6 Skate Multiple Tissues, Normalized *Leucoraja erinacea* cDNA clone LE_MX1_51F10 5', mRNA sequence	464	GH161215.1	97

Table 5. Results of sequence analyses performed with NCBI DNA BLAST and NCBI EST BLAST search

Species	No. of sample	
	NCBI DNA BLAST	NCBI EST BLAST
Insect	23	19
Other	25	29
Total	48	48

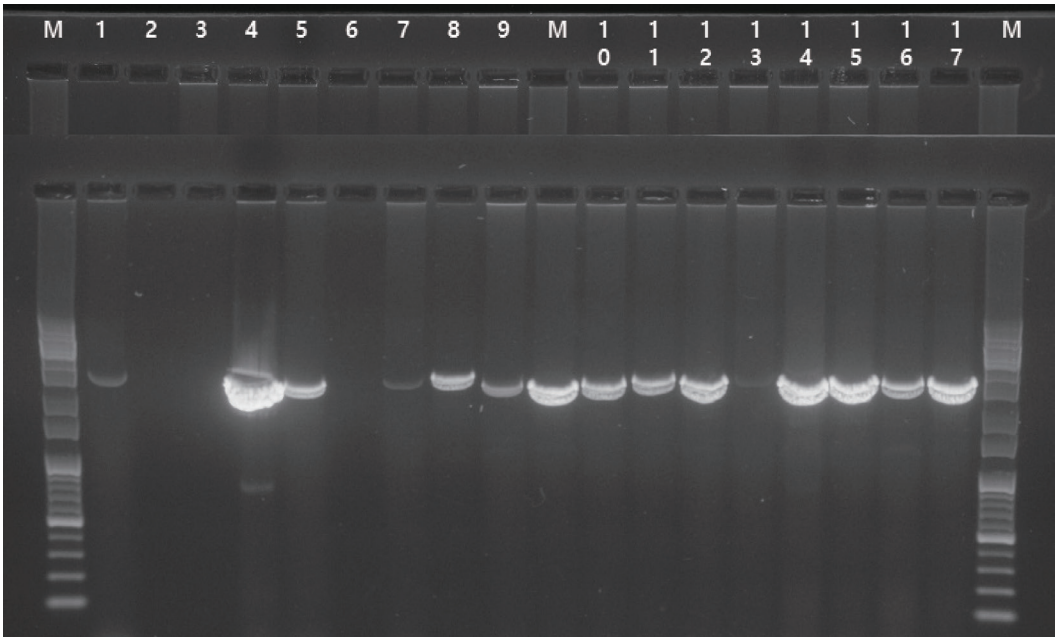

Fig. 7. Electrophoresis of the plasmid DNA cleaved by *Hpa*I. The band was located between 3 kb and 4 kb. M: 1 kb plus DNA ladder marker; Lanes 1 to 48: plasmid DNAs of *Hv1* to 48 were cut with *Hpa*I, except for *Hv18*.

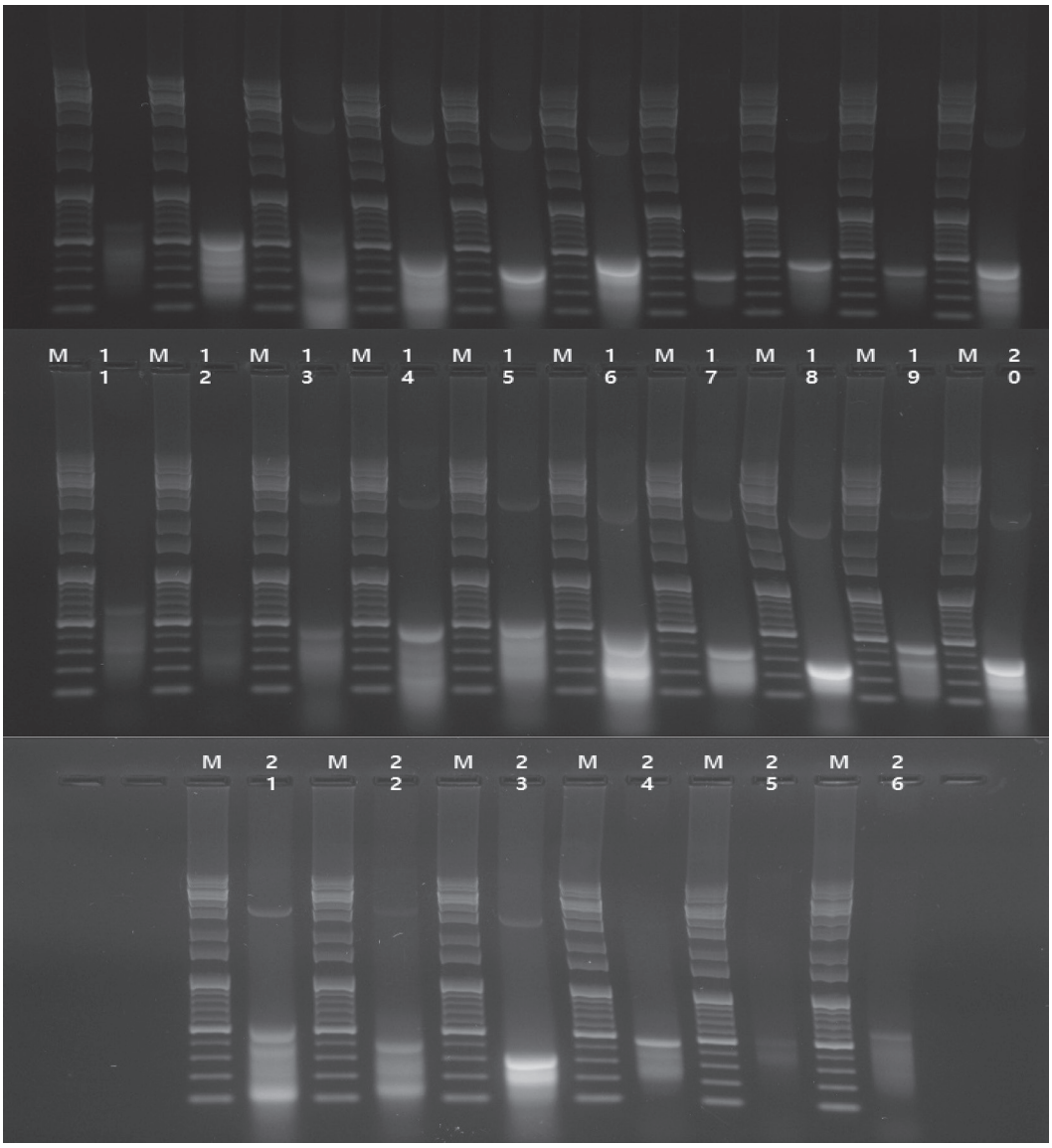

Fig. 8. Electrophoresis of dsRNAs. The bands corresponded to dsRNA sizes between 200 bp and 600 bp. M: 1 kb plus DNA ladder marker; Lanes 1 to 26: 26 synthesized dsRNAs that had sizes varying between 200 bp and 600 bp.

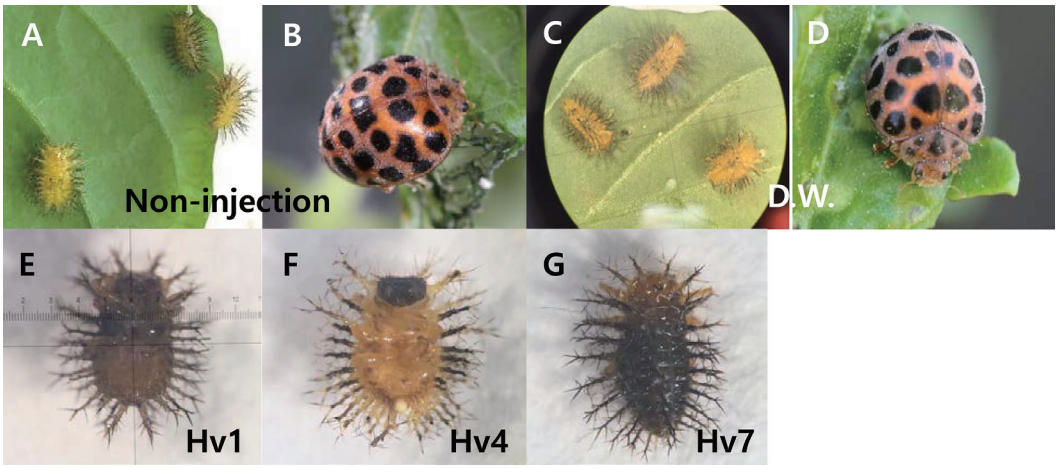

Fig. 9. Phenotype of *Henosepilachna vigintioctomaculata* observed after injection. (A) Un–injected stage; (B) Normally developing adult; (C) Larva injected with distilled water (D.W); (D) Adult developing normally following injection with D.W.; (E) Dead larva following injection with Hv1 dsRNA; (F) Dead larva following injection with Hv4 dsRNA; (G) Dead larva following injection with Hv7 dsRNA.

Table 6. Effect of dsRNA injection on phenotypic variation and mortality of the fourth instar larvae of *Henosepilachna vigintioctomaculata*. Ten larvae were injected with dsRNA for each gene

No.	No. of dead larvae	No. of dead pupae	No. of dead adults	No. of alive adults
Non–injection	0	0	0	10
D.W.	0	0	0	10
Hv1	6	1	0	3
Hv2	2	0	0	8
Hv4	10	0	0	0
Hv5	1	0	0	9
Hv7	7	0	0	2
Hv10	2	0	0	8
Hv11	0	0	1	9
Hv12	2	0	0	8
Hv15	2	0	0	8
Hv16	2	0	0	8
Hv26	1	0	0	9
Hv27	1	0	0	9
Hv28	1	0	0	9
Hv29	0	0	0	10
Hv31	0	0	0	10

DISCUSSION

In recent years, various studies have been conducted using RNAi as a new pest control strategy. For an effective RNAi, the selection of the suitable target gene and the efficient delivery of the dsRNA of the target gene to the target site play important roles (Burand and Hunter, 2013). In fact, previous studies on RNAi have achieved effective RNAi by selecting genes important for the growth and reproduction of insects, such as those encoding various enzymes, receptors, hormones, and housekeeping genes, as target genes for RNAi (Ekert *et al*., 2014; Liu *et al*., 2016; Raza *et al*., 2016; Zhao *et al*., 2008). However, it is difficult to establish the target genes for achieving RNAi in *H. vigintioctomaculata*, as this insect is not a model organism and there is paucity of information pertaining to its genes.

Therefore, in this study, a cDNA library was constructed using the Gateway system for selecting the target genes suitable for RNAi. The Gateway system is a new cloning method that uses specific DNA sequences called att sites and the site–specific recombination system of bacteriophage λ. The efficiency and cloning accuracy of the Gateway system is very high. In this study, the Gateway system was used to efficiently clone various cDNA, since PCR products containing the att site can be easily cloned into a donor vector containing the att sequence (Hartley *et al*., 2000). In this study, total RNA was first extracted from the imagos of *H. vigintioctomaculata* using Trizol. When extracting total RNA using Trizol, higher concentrations of total RNA can be extracted from the same sample than when using RNA extraction kits, although the purity of the RNA extracted is lower than that of the RNA extracted using kits (Ko, 2015). When mRNAs were isolated from the total RNA for synthesizing the cDNAs, a random primer (N20) was used instead of the oligo (dT) primer to obtain cDNA of various sizes. Following cDNA amplification, a primer containing *Eco*RI and *Hin*dIII restriction sites was constructed and was used to subclone sequences containing the attB sequence (for use in the Gateway system) into the LITMUS 28i transcription vector. A band corresponding to sizes between 200 and 600 bp was confirmed by electrophoresis, and cDNA of sizes varying between 100 and 500 bp were obtained, excluding the adapters at both ends. The cDNA containing the att site were cloned into the pDONR207 vector using BP recombination and were then transformed into *E. coli* cells by electro–transformation. The transformant was cultured on a medium containing gentamycin, and the number of growing colonies was counted to measure the titer. The titer of the final cDNA library was 3.15×10^5 cfu/ml.

The resulting cDNA library was cloned into a LITMUS 28i vector using the restriction enzymes *Eco*RI and *Hin*dIII. As mentioned above, it is also important to efficiently deliver the dsRNA into the bodies of the insects for an effective RNAi. The dsRNA delivery methods include dsRNA injection, feeding with artificial diets containing dsRNA, feeding a diet of transgenic plants expressing dsRNA, and using plants expressing dsRNA through VIGS (Yu *et al*., 2013). Among them, the dsRNA injection method is labor intensive because the dsRNA thus synthesized need to be individually injected into the bodies of insects. However, it can be easily carried out in most laboratories, and the effects of RNAi can be suitably identified for non–model species as well as for model organisms such as *Tribolium castaneum* and insects belonging to Drosophilidae, which have been mainly used for RNAi studies. Moreover, its advantage lies in the fact that it allows the effect of RNAi on the desired developmental stages to be studied selectively (Bell'es, 2010). In this study, the genes from the cDNA

library were cloned into the LITMUS 28i vector, which has two T7 promoters and can therefore be used to synthesize the dsRNAs from the inserts. Subsequent treatment with the restriction enzymes *Eco*RI and *Hin*dIII confirmed the diversity and information of the cloned genes, and the genetic information was determined from the size of the insert, by sequencing, and from the NCBI BLAST search. Inserts of sizes varying between 100 and 500 bp were identified, which did not have duplicated genes. Most of the genes identified belonged to the order Coleoptera, such as *T. castaneum*, *Tomicus piniperda*, and *Leptinotarsa decemlineata*, or to insects of other taxa. These results suggested that there have been few studies on the function of the genes of *H. vigintioctomaculata*, and owing to the lack of genetic information pertaining to *H. vigintioctomaculata*, the results identified similarities with genes of other species of Coleoptera.

After confirming the gene information of the dsRNA synthesized from the 48 inserts, dsRNAs of sizes between 200 and 600 bp, suitable for RNAi, were synthesized. There are a number of factors that influence the effect of RNAi, including the length of the dsRNA. In the case of *Diabrotica undecimpunctata howardi*, dsRNA of varying lengths was synthesized for the Snf7 gene, which has a size of 240 bp, and the lethal effect was investigated by oral feeding. The dsRNA of sizes less than 60 bp showed negligible lethality, while dsRNA of sizes varying between 60 and 240 bp produced mortality rate of 95% or more (Bolognesi *et al*., 2012). Similar results were obtained when dsRNAs of sizes 21–594 bp were used for RNAi, while targeting the luciferase gene of the firefly in the S2 cell of *Drosophila*. With the increase in the length of the dsRNAs, the expression level of the target gene decreased. The gene expression level gradually decreased with time when dsRNAs of 1000 bp were used; however, the expression changed only slightly when the size of the dsRNA was 21 bp. Thus, to analyze the expression level of the target gene over time, dsRNA of sizes varying between 21 bp and 1000 bp were used in that study (Saleh *et al*., 2006).

In this study, the dsRNA synthesized from the genes in the cDNA library were injected into the abdomen of the fourth instar larvae of *H. vigintioctomaculata*, and most of the larvae injected with the *Hv1*, *Hv4*, and *Hv7* genes died. Additionally, the larvae that died naturally did not display any change in body color. However, body color in the larvae injected with the dsRNA of all the three genes gradually turned black and the larvae subsequently died.

The three genes, *Hv1*, *Hv4*, and *Hv7*, that caused larval mortality, had 79% similarity with the cytochrome P450 of the Chinese alligator, 85% similarity with a gene of the parasite *Trypanosoma cruzi*, and 80% similarity with the RING finger protein gene of *Anoplophora glabripennis*. Cytochrome P450 is a reduced type of protoheme–containing protein enzyme with an absorption spectrum that shows relative maximum value in the vicinity of 450 nm by bonding to CO. It catalyzes almost all the monooxygenase reactions in lipid metabolism and reduces the efficacy and toxicity of drugs by adding one atom of oxygen to the drug and environmental pollutants in the microsomes of animal cells. The overexpression of the P450 CYP6CM1 gene of *Bemisia tabaci* has been reported to be associated with increased drug–resistance in insects (Karunker *et al*., 2008). The suppression of gene expression through RNAi has been shown to increase the rate of insecticidal activity against imidacloprid (Li *et al*., 2014). The RING finger protein is generally known to function as a ligase by binding to the ubiquitination enzymes in the ubiquitylation pathway. Specifically, it maintains a balance between cell proliferation and apoptosis and is involved in the apoptotic pathway. The absence of the RING finger protein is considered to be associated with growth control disorders (Joazeiro and Weissman, 2000). The RING finger protein has also been found in other insects, including *Aedes aegypti*, *Acyrthosiphon pisum*, *Apis* sp., and *T. castaneum*.

In a study on *Trachelizus bisulcatus*, 24 genes were injected into the larvae to select the target genes to be used in RNAi, and 12 genes whose suppression was associated with high mortality, were selected. The dsRNA soaking assay was performed with the 12 genes selected by injection experiments. Based on the results of the injection experiments and the soaking assay, three genes, *prosα2*, *RpS13*, and *Snf7*, were finally selected and fed through an artificial feeding system. All the three genes showed a 50% or greater decrease in gene expression, and an insecticidal rate of nearly 70% was achieved, which demonstrated their effect on insect pest control (Prentice *et al*., 2017). For *Rhynchophorus ferrugineus papuanus*, RNAi was induced in the fifth instar larvae through injection, artificial feeding, and topical application, for silencing the catalase gene. As a result, when 5 μl of catalase dsRNA was injected into the abdomen of the larvae, maximum effects were observed, with a growth inhibition rate of 123.5% and an insecticidal rate of 80% (Al–Ayedh *et al*., 2016). In a study on *Harmonia axyridis*, the dsRNA of the ecdysone receptor gene involved in ecdysis was injected into the fourth instar larvae. As a result, most of the larvae injected with dsRNA died within 4 days after injection without metamorphosing into pupa. The pigment of the integument was not expressed in the the dorsal thoracic region of some of the larvae (Jung, 2016).

Thus, the delivery of dsRNA by injection is suitable for screening and selecting the most suitable target genes from among the different genes, and can induce gene knockdown faster than the other dsRNA delivery methods. However, since the delivery of dsRNA by injection is not likely to maintain gene knockdown for a long time in most insects, it is not practical for outdoor applications. Therefore, to maintain the effect of the RNAi, a method for continuously delivering dsRNA and maintaining a constant level of dsRNA in the insect body—such as by using a transgenic plant that expresses dsRNA or using a plant that is made to express dsRNA through VIGS—is essential. Various studies have been conducted to develop a suitable

method for this purpose (Das *et al*., 2015; Mao *et al*., 2010; Mao *et al*., 2011; Ko and Youn, 2015).

This study was conducted to confirm the effect of RNAi on *H. vigintioctomaculata*. First, for selecting the gene to be targeted by RNAi, a cDNA library pertaining to *H. vigintioctomaculata* was constructed, using the Gateway system, and a final cDNA library having a titer of 3.15×10^5 cfu/ml was obtained. The resulting cDNA library was cloned into a LITMUS 28i vector to synthesize dsRNA that were injected into the abdomen of the fourth instar larvae of *H. vigintioctomaculata*. As a result, three genes, namely, *Hv1*, *Hv4*, and *Hv7*, were identified, the silencing of which showed high insecticidal effect. Thus, screening genes from cDNA libraries by injecting dsRNA enables the selection of genes suitable for RNAi. Moreover, it allows the function of the gene to be analyzed by studying mutational changes in the phenotype, in addition to controlling the insects through RNAi.

AUTHOR CONTRIBUTIONS

Jeong Hee Kim designed the study, performed the effect of RNAi by injecting dsRNAs and constructing a cDNA library for selecting the genes to be targeted by RNAi, the comprehensive experiments, analyzed the data and wrote the paper. Hye Ri Kwon participated in the construction of the cDNA library for selecting genes to be targeted by RNAi. Yong Man Yu edited the paper. Chisa Yasunaga–Aoki participated in the design of the study and discussed on 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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