Knock down of molt regulating gene for development control of *Helicoverpa armigera*

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ABSTRACT. RNA interference (RNAi) caused by exogenous double-stranded RNA (dsRNA) is a powerful tool, and to date is widely adopted to knockdown genetic targets crucial for growth and development of agriculturally important insect pests. *Helicoverpa armigera* is a pest feeding on more than 30 economically important crops worldwide and a major threat. Resistance to insecticides and Bt toxins is gradually increasing in the field. RNAi mediated knockdown of *H. armigera* genes by producing their dsRNA homologous to genetic target in bacteria has a high potential for insect management. A steroid signaling genes *Ecdysone receptor (EcR),* of *H. armigera* was selected as genetic target. Three different fragments comprising of a coding sequence of <450bp were cloned into the L4440 vector for dsRNA production in bacteria. The most effective fragment was further analyzed for post feeding effects on larval growth and development. After ingesting bacterial expressed dsRNA, mRNA levels of the target EcR gene declined dramatically resulting larval mortality and an abnormal development. Analysis of transcripts level by quantitative RT-PCR revealed that larval lethality was attributable to the knock down of genetic targets by RNAi. Results demonstrated that down regulation of *H. armigera* genes involved in transcriptional stimulation of development gene has aptitude as a bio-insecticide to control *H. armigera* population sizes and therefore decrease crop loss.

Keywords: RNA interference; Double-stranded RNA; *Helicoverpa armigera;* Bt toxins; Gene regulation; Gene silencing

INTRODUCTION

Protists, fungi, plant and animal have developed defense mechanism based on sequence specific breakdown of double stranded RNA against invading genetic elements. These mechanisms entail similar degradation processes- known as quelling in fungi, RNA interference (RNAi) in animals and post transcriptional gene silencing in plants (Meister et al., 2004). The phenomenon of an RNA-silencing was firstly reported by Jorgensen and co-workers (Napoli et al., 1990), who surprisingly observed transgenic petunia-mediated gene knockdown while trying to over-express the chalcone synthase gene to enhance flower coloration. RNA interference approach has been extensively exploited in functional genomics research on insects is rooted in the expression of dsRNA that has almost 100% sequence similarity with the desired target gene in order to ensure optimal knock down efficiency (Push et al., 2003; Belles et al., 2010). This mechanism was first reported in Caenorhabditis elegans by knock down of *unc-22* gene in an efficient and particular manner (Fire et al., 1998). So far, many advancements have been accomplished in this area. Substantial advancement has been made in understanding to interpret the RNAi mechanism and its practical application and is a naturally existing defense mechanism in eukaryotes against viruses and also serve as a regulation of gene expression controlled by external and internal ambience circumstances (Voinnet, 2001). RNAi derived gene silencing depends on the extrinsic encoding of short interfering RNAs/ microRNAs by an organism to regulate gene expression. This mechanism can also be prompted in insect by importing dsRNA into cell by a carrier protein such as systematic RNA interference defective-1(SID-1) resulting in down regulation of genetic target (Scott et al., 2013). RNAi has affirmed its effectiveness in functional genomic studies conducted on pests, and due to high specificity, it has been considered a valuable approach to control agriculturally important insects (Huvenne and Smagghe, 2010). Recent advancement in pest management strategies reduces the harmful effect caused by insect damage to economical crops. *Helicoverpa armigera*, a voracious feeder of more than 30 species of economically important crops has been controlled worldwide by encoding an insecticidal protein in plant acquired from Bacillus thuringiensis (Bt) (Onkaramurthy et al., 2015). These transgenic crops relieved the agricultural sector providing protection form loss caused by insect/pest (Carriere et al., 2010).

Due to its widespread hosts, numerous generations, migratory behavior, high fecundity, and insecticide confrontation, *H. armigera* has become a complicated insect/pest to control. Globally, *H. armigera* developed resistance against a wide range of insecticides, also in Pakistan (McCaffery, 1998). The repeated and arbitrary application of insecticides has resulted in the commencement of resistance in numerous insect populations (Ferre and Van, 2002). Notably the application of Bacillus thuringiensis (Bt) crops has modernized crop production worldwide. Conversely, Bt cotton crops may not be the possible way out for all pest problems (Sayyed et al., 2008). Additionally, reports from India and China revealed the resistance against Cry1Ac is increasingly observed in some population of *H. armigera* (Liu et al., 2010; Tabashnik et al., 2009). Delivery of dsRNA regulates expression of two genes Ap-cath-L and Ap-crt that encodes cathepsin-L and calreticulin-L respectively in pea aphid Acyrthosiphon pisum (Zhang et al., 2010). RNAi triggered gene silencing in mid-gut genes (Nlaub and Nlsid-1) of Nilaparvata lugens established effective role of RNAi for governing harmful insects in the field (Joge et al., 2016). Knock down of two chitin synthase genes (AgCHS1 and AgCHS2) resulting dEcReased chitin level in the larvae of African malaria mosquito led to the increased susceptibility to the insecticide, diflubenzuron (Brodersen and Voinnet, 2006). Several delivery strategies for the use of RNAi in crop protection have been proposed, including spraying, GM plants, VIGS and other in planta applications (Hileman et al., 2005). This siRNA can also guide gene knock down through chromatin rearrangements or hamper of translation (Liu et al., 2010). The nuclear receptor complex, ecdysone receptor (*EcR*) and ultraspiracle (USP), principally control insect metamorphosis and molting process in conjunction with 20-hydroxyecdysone (20E) hormone (Koelle et al., 1992). RNAi mediated gene silencing of *EcR*-USP induced significant mortality and molting abnormalities in many insect pests (Matzke et al., 2001; Holen et al., 2002; Reynolds et al., 2004; Luo et al., 2004). Since *EcR*-USP is important for insect development and growth, we presume that targeting *EcR* through bacterially expressed dsRNA might be productive to improve insect control. In

this report, three different fragments of insect associated gene were used to target *EcR*. The results demonstrate that silencing pest genes through bacterially expressed dsRNA is able to improve insect control.

MATERIALS AND METHODS

H. armigera culturing and feeding

H. armigera larvae were collected from different geographical regions of Punjab, Pakistan and maintained at the Entomology Lab, Centre of Excellence in Molecular Biology, of the University of The Punjab. *H. armigera* were fed on artificial diet and adults were maintained in rearing cages (30 × 20 × 20 cm) and fed with 10% (honey/water) honey solution. The eggs were collected each successive day and placed on an artificial diet at 26°C and 60% relative humidity and allowed to hatch. *in-vivo* feeding assays was performed with newborn larvae. Each larva was placed in a box containing artificial diet coated with dsRNA.

Total RNA isolation and cDNA synthesis

Total RNA isolation from the collected samples of 3rd star larvae was carried out using TRIzol reagent (Life Technologies cat no. 15596026). The homogenate of larvae tissue samples was made with 1 mL of TRIzol reagent and incubated at room temperature (RT) for 5 minutes. 200 µL of chloroform was added and content was mixed vigorously for 15 seconds at incubated at room temperature for 10m minutes followed by centrifugation for 5 minutes at 13,000 rpm (at 4°C). The upper clear phase was transferred to another nuclease free 1.5 mL Eppendorf tube and 500 µL of pre-chilled isopropanol was added and incubated for 10 minutes at room temperature followed by centrifugation at. Washing of the pellet was carried out using 500 µL of 75% ethanol and centrifuged again at 13000 rpm (at 4°C) for 15 minutes (2x) and resuspended in 20 µL DEPC water. The cDNA was synthesized using 1 µ g of Total RNA template from each larval sample using Revert Aid First-Strand cDNA synthesis kit (Thermo Fisher Scientific, cat no. 1622) following the suggested practice.

Amplification of targeted genes

Amplification of the targeted gene was carried out using gene specific primers (S1) and DreamTaq Green PCR Master mix 2x (Thermo Fisher Scientific, cat no. K1081). The PCR reaction contained 10 µ-L of DreamTaq Green PCR master mix, 0.8 µ-L template cDNA and 0.4 µ-L of both forward and reverse primers (10 µ-mol) (S1) added to PCR tube (0.2 mL) and nuclease free water to final volume of 20 µ L. The PCR product was electrophoresed on 1.2% agarose gel and then subjected to sequencing. The reverse compliment of the obtained nucleotide sequence of target genes was analyzed using RNAi Designing Tool (BLOCK iT™ RNAI Designer) to select the most optimal region. The most efficient gene fragment edited to replace A from ATG (start codon) with G to prevent the start of translation. The 405bp, 426 bp and 446 bp gene fragments (g Blocks® Gene Fragments, IDT) (S1) of *EcR* and 379 bp *of egfp* were synthesized respectively.

Plasmid assembly and dsRNA expression

For the design of the L4440 plasmid to express dsRNA, g Blocks® Gene Fragments of *EcR* and *egfp* genes were amplified by PCR harnessing specific primers. The sequence is encompassed with Sma1 and HindIII sites to enable cloning into the multiple cloning site (MCS) of L4440 vector and '*egfp*' is employed as a control. L4440 plasmid was obtained from AddGene (Plasmid # 1654) and contain two promoters having an inverted orientation and flanked by MCS. Verification of constructs including *L4440-EcR* and *L4440-egfp* was performed by PCR, a restriction method and sequencing. Competent cells which lack RNase III were developed treating CaCl2 and later, transformation was done with the genetically engineered plasmid. Then, one colony is chosen from the agar plate and cultured in LB at 37°C overnight and shaking at 220 rpm. Dilution of culture was performed up to 100 times in 800 ml 2×YT accompanied with 75 µg/ml and 12.5 ug/ml of ampicillin and tetracycline respectively at 37°C with optical density OD600=0.5. T7 polymerase was induced by 0.4mM IPTG and additional incubation to bacteria was done at 37°C for 4 h.

Quantification and purification of dsRNA

Total Nucleic Acid was extracted as illustrated by Timmons et al., 2001. The culture of bacteria was centrifuged at 5,000 g for 10 min. The bacterial pellet was resuspended in I M acetate or 10 M EDTA supplemented with the exact volume of phenol:chloroform:isoamylalcohol (25:24:1). Then incubation of samples was done at 37°C and they were then centrifuged at 12,000g for 15 min. The upper transparent layer was mixed with isopropanol and kept at -20°C overnight. Next the samples were centrifuged at 12,000 g for 30 min. Samples were mixed with RQ1 RNase free DNase to eliminate ss DNA and then with RNase to remove ssRNA. The dsRNA pellet was resuspended in Tris-EDTA solution (1X) pH 7.5. Resuspended dsRNA was loaded onto 1.2% agarose gel treated with ethidium bromide and visualized under UV light. The concentration was measured with a NanoDrop 1000 (Thermo scientific).

Persistence of dsRNA in artificial diet and gut

For the evaluation of dsRNA in the midgut, feeding of purified dsRNA for three days was performed and then a midgut section was dissected, and dsRNA was extracted. The stability of dsRNA throughout the feeding process was evaluated on 1.2% agarose gel TAE. Extraction of dsRNA from diet was also done and evaluated on 1.2% agarose TAE gel.

Feeding bioassays

Newly hatched larvae of *H. armigera* were transferred into a 30 cm × 30 cm × 30 cm box. Thirty larvae were selected for each analysis and test repeated 3 times. Larvae were starved for 4 hours prior to *in-vivo* feeding experiments in order to maximize the consumption diet. Synthetic diet was prepared and cut into small pieces of 3 cm^2. Each exposed side of diet was coated with 100 µl of dsRNA solution. Pieces of artificial diet containing dsRNA were replaced twice a day. *H. armigera* were nourished on the diet for 10 days constantly and after that were maintained on an artificial diet without dsRNA to evaluate post feeding effects. Collection of individuals was preceded on days 2,4,6 8 and 10 to conduct qRT-PCR. But before qRT-PCR individuals were kept in liquid nitrogen and stored at -80 c. TRIzol reagent (Life Technologies cat no. 15596026) was used to extract RNA from larvae and death rate was noted on each successive day up-to day 10.

Quantification of gene expression by RT-qPCR

Larvae were collected on day 0, 2, 4, 6, 8, and 10 days after their release to feed on diet coated with dsRNA. TRIzol reagent was used to extract Total RNA according to the standard protocol. Extracted RNA was quantified using Nanodrop and 1 µg of total RNA was subjected to cDNA synthesis according to the manufacturer's instructions (First Strand cDNA Synthesis kit Thermo Fisher, USA # K1612). Expression of targeted genes in *H. armigera* larvae fed on diet coated with dsRNA was quantified by RT-qPCR technique. The 20 µL reaction mixture contained 1 ul of cDNA 0.5 µL of both forward and reverse primers (10 pmole) (S1), 18S ribosomal RNA and β-Actin were used as an internal control.

The thermal profile of qRT-PCR mixture was: 94°C for 5 minutes followed by 40 cycles, comprising 94°C (for 30 seconds), 60°C (for 30 seconds) and 72°C (for 30 seconds). The RT-qPCR was performed using PikoReal™ Real-Time PCR System and each reaction was run in triplicate. Melt curve analysis was performed at the end of each reaction to evaluate the specificity of the amplification product from 60-85°C with an increase of 0.5°C after every 10 seconds. 2-ΔΔCT approach was used to analyze quantification results (Livak and Schmittgen, 2001). Standardization of consequent CT values was carried out using β-Actin and 18S ribosomal RNA as reference gene. The gene expression level was calculated in different larval groups and the fold variation in the transcript levels of targeted genes was determined as described previously.

The data collected from above mentioned experimental replicates was subjected to SPSS 17 for one-way analysis of variance (ANOVA) and Duncan's test was employed to assess the significant difference between different test groups. $p<0.05$ was considered as significant.

RESULTS

A molt regulating hormone (*EcR*) gene was selected as our genetic target to evaluate its efficacy in pest management. A 627 bp partial CDS sequence was obtained (GenBank accession number: MG457282) using gene specific primers (Table 1). The effect of RNAi is precise, but the biological hazard of dsRNA induced RNAi yet prevails because of possible non-target silencing of the highly identical homologous genes (Zhu et al., 2012). We targeted molt regulating physiological gene as it is one of the crucial process of pest growth and development which is widely targeted to control insect pests. Data obtained from field trials revealed that various chemistries that target molting process are selectively lethal to lepidopteran insects and valuable insects are safe (Zhao et al., 2004). Therefore, we concentrate to target pest-specific molting gene *EcR*, which controls tissue specific metamorphosis associated gene expression to induce RNAi. During RNAi process, the siRNA work as a template for breakdown of homologous transcription together with the RISC complex (Matzke et al., 2001). After binding of mRNA with the RISC, variation in knockdown efficacy can be observed, since different siRNAs can attach to the same mRNA at different sites (Holen et al., 2002; Reynolds et al., 2004).

Table 1. A 627 bp partial CDS sequence was obtained (GenBank accession number: MG457282) using gene specific primers.

Primer Name	Sequence 5′ to 3′	Purpose
EcR-F	TCCAGCTTCAAGTGTGAACG	Amplification
EcR-R	CATATCCGAGTCCTCGTCGT	Amplification
L4EcR-1-F	GGCAAGCTTCAGCACAGAGGGCGAGGCGA	L4440 cloning
L4EcR-1-R	GGCGAGCTCCCCGGAGGATCACATTGCAT	L4440 cloning
L4EcR-2-F	GGCAAGCTTGAAGAGCTATGTCTAGTCTG	L4440 cloning
L4EcR-2-R	GGCGAGCTCCTTCAATCTGTTCTGTTCCA	L4440 cloning
L4EcR-3-F	GGCAAGCTT GGCAATGCTTGCGGAATGGA	L4440 cloning
L4EcR-3-R	GGCGAGCTCATATCCGAGTCCTCGTCGTC	L4440 cloning
L4egfp-F	GGCAAGCTTCACATGAAGCAGCACGACTT	L4440 cloning
L4egfp-R	GGCGAGCTCTGCTCAGGTAGTGGTTGTCG	L4440 cloning
qEcR-F	GGTGCTCGACTCACTCTTCC	qRT-PCR
qEcR-R	CTGACATCGGAGGTGCAGTA	qRT-PCR
qegfp-F	AGAACGGCATCAAGGTGAAC	qRT-PCR
qegfp-R	TGCTCAGGTAGTGGTTGTCG	qRT-PCR
q18s-F	GCCTCTTTGTCCAGATCAGC	qRT-PCR
q18s-R	ACTTCTTGGCTTTGGCAGAA	qRT-PCR
qβAct-F	TGCGTGACATCAAGGAGAAG	qRT-PCR
qβAct-R	TACCGATGGTGATGACCTGA	qRT-PCR

In the present study, different regions of the *EcR* were selected to produce dsRNAs in bacteria, and after ingestion by insect, it was assumed that each of the dsRNA will be processed into no of siRNA and bind with RISC complex. Varying degree of larval mortality was observed in different dsRNA. This varying efficiency of RNAi can be explained on the basis of various hypotheses proposed so far, such as structure of targeted local transcript, sequence specific phosphorylation of mRNA and the positional effects of local protein (Luo and Chang, 2004).

The accessibility and secondary structure of target mRNA may the principal factor influencing knockdown efficacy (Shao et al., 2007), because different secondary structures of mRNA have dissimilar recognition or binding efficacy for guide siRNAs. Similar results were reported by Zhang et al., 2012 who selected two different fragments of CiHR3 for *in-vivo* feeding analysis, and their finds revealed that significant lethality was observed with CiHR3-I2 dsRNA rather than CiHR3-I1. The results of another study conducted by Xiong et al., 2013, using 4 different dsRNAs to target HaHR showed that HaHR3 dsRNA treated group showed enhanced larval deformity and mortality compared to others. Our findings of siRNA binding also showed the probability of varying degree of dsRNA efficacy.

Molecular cloning of partial CDS of Ec-R and stability of dsRNA in diet

The target gene was amplified using RT-PCR. The amplified partial CDS obtained for the target *Ec-R* gene was 627 (GenBank accession number MG457282) to which the Blastn results showed high similarity of 96%.

Obtained nucleotide sequence was analyzed using RNAi tool by Thermofischer to determine the optimal dsRNA fragment (≤ 450 bp). The resulting fragments comprising *EcR*1 (405 bp), *EcR*2 (426 bp) and *EcR*3 (446 bp) (S1) were cloned in antisense orientation into the L4440 dsRNA vector and expressed in HT115 cells by inducing with 0.04 M IPTG. The dsRNA was extracted and purified by using the method prescribed by Fire et al., (36). The dsRNA was extracted from artificial diet to evaluate its stability and run on 1.2% agarose gel. The results of gel electrophoresis demonstrated that dsRNA remain stable in diet even after 10 hours of addition (Figure 1).

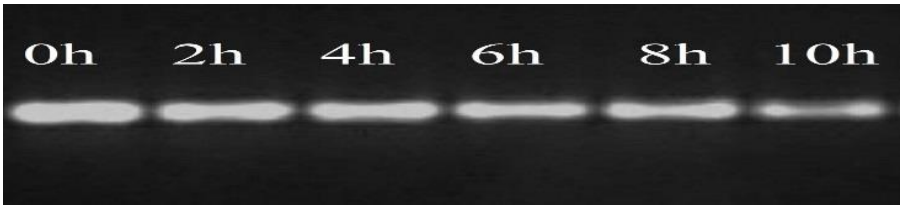

Figure 1. Stability of dsRNA on artificial diet. dsRNA on artificial diet was extracted and examined by Agarose gel electrophoresis for integrity at different intervals of time.

Ingestion of bacterially expressed dsRNA induced RNAi in *H. armigera*

After bacterial transformations with the cloned L4440 into HT115 bacteria, total nucleic acid was isolated. Agarose gel electrophoresis showed that each of the cloned fragments were expressed and isolated effectively (Figure 2). After ingesting synthetic diet containing dsRNAs for 10 days, the *in-vivo* bioassay revealed that three different dsRNAs directed against *EcR* increased *H. armigera* larval mortality. Enhanced mortality level was observed after 3 days of feeding *dsEcR*-1 and *dsEcR*-2: after 3 days of feeding, the larval lethality feeding with *dsEcR*-1 and *dsEcR*-2 showed significant differences compared to the *dsEGFP*. After 4 days of feeding, Significant differences were observed among all the dsRNAs targeting *EcR* and *dsEGFP* groups ($P<0.05$). On day 7-9, larval lethality plateaued. The highest level of mortality was observed in larval group treated with *dsEcR*-2 compared to the *dsEGFP* starting on day 4. After continuous feeding with artificial diet containing *dsEcR*-2, mortality was increased up to 46% compared to the *dsEGFP* (13%). The other two fragments failed to enhance mortality after day 6. After 10 days of feeding, reduced mortality rate of 32% and 28% was recorded in *dsEcR*-1 and *dsEcR*-3 groups respectively (Figure 3A). Moreover, significant reduction in body weight was also recorded in *dsEcR* treated group compared to the controlled group (Figure 3B).

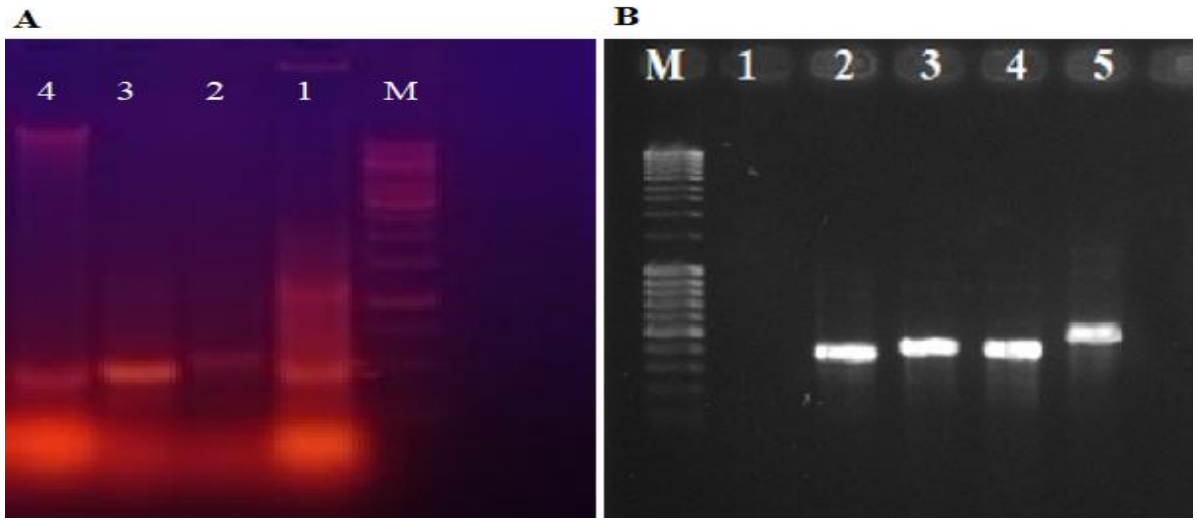

Figure 2. Isolation of *EcR* and *EGFP dsRNAs* fragments expressed in bacteria. (A) Total nucleic acid isolated from transformed bacteria. Loading sequence of the samples in 1.2% agarose gel is, M=1 kb DNA Marker, Lane 1=EGFP, Lane 2-4=EcR (B) Purified dsRNA extracted from bacteria. Loading sequence of the samples in 1.2% agarose gel is, Lane 1= 1Kb DNA Marker, Lane 2=dsEGFP, Lane 3-4=*dsEcR* 1-3

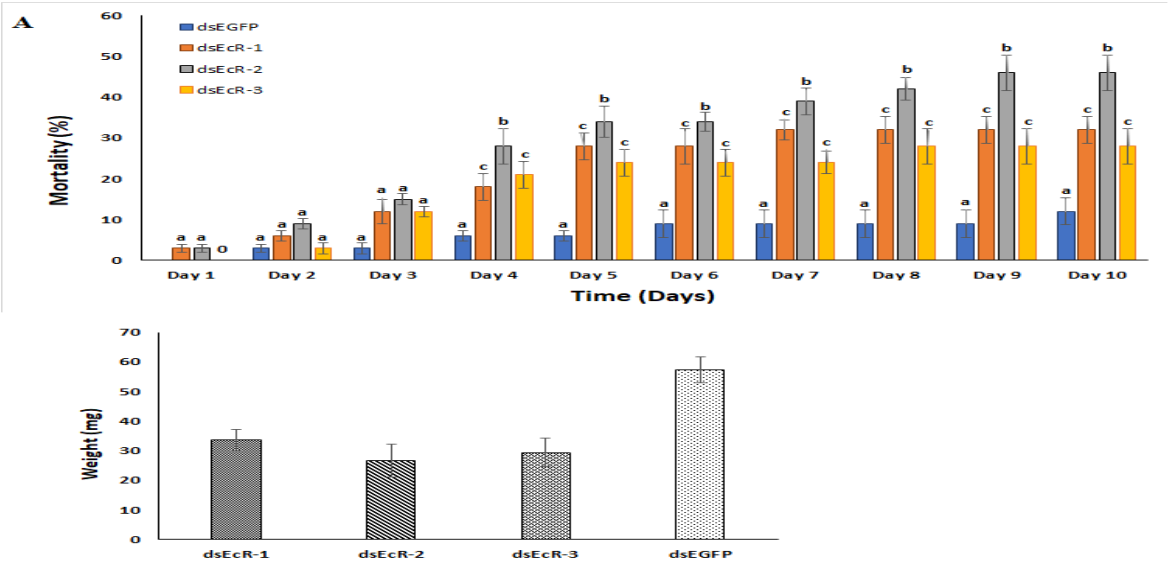

Figure 3. Lraval mortality and body weight (A) Larval mortality in different experimental groups after feeding 10 days. *dsEGFP* used as a control, and *dsEcR* fragments 1-3 (B) Larval body weight in different experimental groups Different letters above standard deviation bars indicate significant difference. All experiments were triplicated. $p<0.05$ was considered as significant (Duncan's test).

Feeding of bacterially expressed dsRNA induced RNAi in *H. armigera*

Among three different groups fed with dsRNAs directed against *EcR* coated diet all of the three groups showed substantial depletion of targeted *EcR* transcripts compared to the control group fed with *ds-egfp* on certain days respectively (Figure 4). All of the three fragments showed effective gene knockdown compared to the *dsEGFP* group, and no significant difference was observed among all the three dsRNA treated groups. The maximum depletion of each targeted genes in larvae fed on dsRNA coated diet ranges from 58% to 96%. The lowest level of transcript was observed on day 10 in the caterpillars treated with *dsEcR*-2, with an 96% reduction as compared to the control *ds-egfp* group. The *EcR* mRNA showed significant down-regulation on both day 4 and day 8 with 89% depletion compared to control group. The significant difference between *dsEcR*-1 and *dsEGFP* was observed on day 4-10 and steady suppression of gene expression (93%) was observed compared to the *dsEGFP* group. After feeding with *dsEcR*-3 fragment for 10 days, the *EcR* gene was successfully depleted (89%) compared to the *dsEGFP* group.

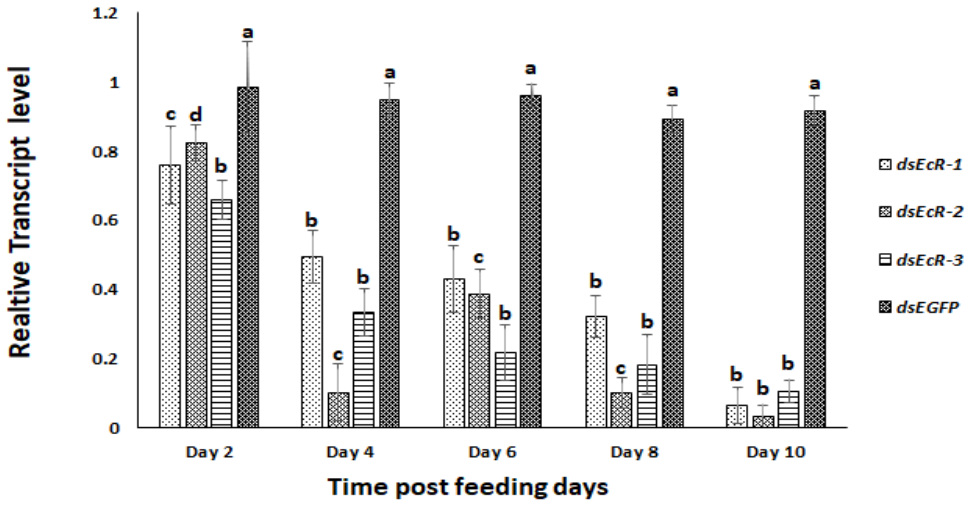

Figure 4. The relative transcript level of *EcR in H. armigera* larva. The larval samples from each group were collected on different days. The *dsEGFP* was used as a control. Different letters above standard deviation bars indicate significant difference. All experiments were triplicated. $p<0.05$ was considered as significant (Duncan's test).

dsRNA specific to *EcR* possess molt inhibiting activity against *H. aramigera*

Previously, it was observed that that oral delivery of bacterially expressed dsRNA induced RNAi effects in *S. exigua* (Tian et al., 2010). Another study revelaed that micro-injection of *dsRNA directed against EcR* resulted in substantial developmental abnormality in *B. mori* (Tian et al., 2010). Therefore, we evaluated whether ingesting *EcR* dsRNAs has potential to interfere metamorphosis in *H. armigera* larvae. It was speculated that oral delivery of dsRNA specific to *EcR* might cause abnormal development in lepidopteran insects. Therefore, larvae fed on *dsEcR* coated diet were maintained on artificial diet after exposure to dsRNA until pupation and adult emergence to evaluate post feeding effect of dsRNA. Although, down regulation of targeted transcripts could be detected by the qRT-PCR but negative effect on growth, development and phenotype was not found by ingesting dsRNA for day 10 except in case of larval groups fed with *ds-EcR* coated diet. The larvae fed with *ds-EcR* coated diet showed slow or no movement and their development was seized compared to control group. Besides mortality, a developmental abnormality at metamorphosis was also recorded in the *ds-EcR* treatment group. Apparently, larval development and growth was not disturbed by *dsEGFP* in *H. armigera* larvae. The control group treated with *dsEGFP* dsRNA caused less than 8% larval lethality. However, the group treated with *dsEcR*-2 showed more than 63% abnormal phenotype. (Figure 5). Most of the caterpillars fed with *dsEcR*-2 coated artificial diet failed to remove old cuticle during larval metamorphosis or having larval pupal intermediate phenotype. The delayed growth resulted in reduced body size and body weight of the larvae fed with *dsEcR*-2 (Figure 5).

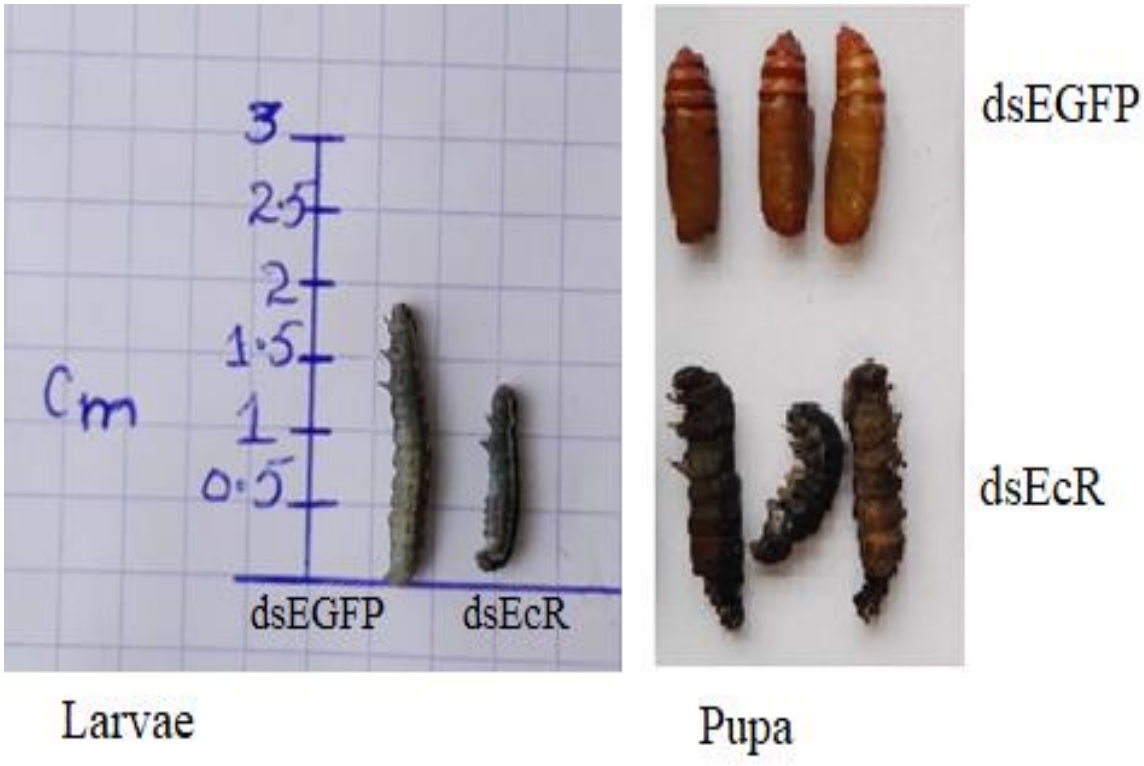

Figure 5. Larval body size and abnormal phenotype development in *Helicovera armigera* fed on bacterially expressed *dsEcR*. Larval body Size and Inhibition of pupation in *H. armigera* fed on *dsEcR* compared to the *dsEGFP*.

Delayed development in *H. armigera* larvae fed with *dsEcR*

Since the larval group treated with *dsEcR*-2 induced higher level of mortality and abnormal phenotype, delayed growth and development was also observed compared to the control group. The larvae fed with *dsEcR*-2 molted into pupae on day 20-29 compared to the control group in which pupation completed on day 23 (Figure 6).

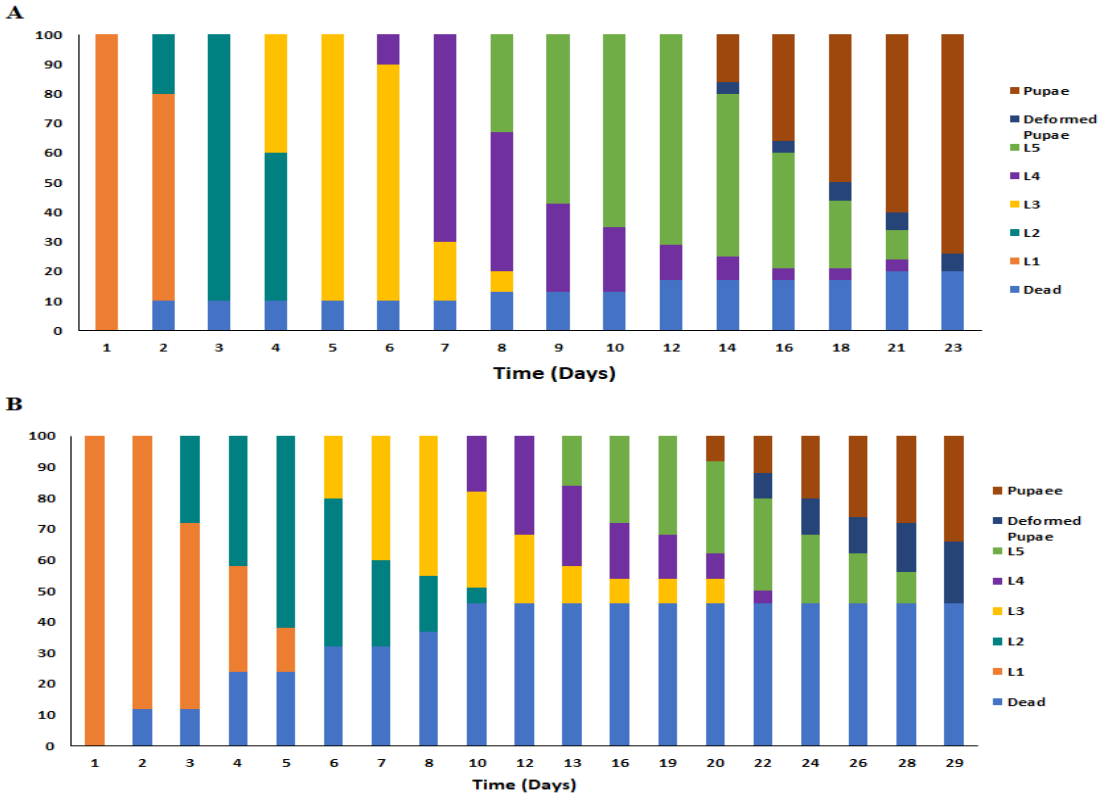

Figure 6. larval time taken for pupation in *dsEGFP* and *dsEcR* groups. The experimental larvae were maintained on artificial diet after 10 days of oral delivery of dsRNAs. The larvae treated with *dsEcR* took longer time to molt into pupa and also showed larval pupal intermediate phenotype.

H. armigera larvae fed on *dsEcR* die with lethal molting defects

Concurrently, *in-vivo* feeding bioassay with *dsEcR*-2 treated larval group showed significantly reduced and delayed growth in the *H. armigera* larvae. The body size was reduced significantly because of longer time taken to molt compared to the *dsEGFP* control group. Moreover, significant larval mortality was observed during metamorphosis in *dsEcR* group (37%) compared to the control (8%) (Figure 7). Conclusively, larval group treated with *dsEcR* revealed thar targeting molt regulating *EcR* gene could induce larval mortality, abnormal phenotype and inhibit adult emergence form pupa.

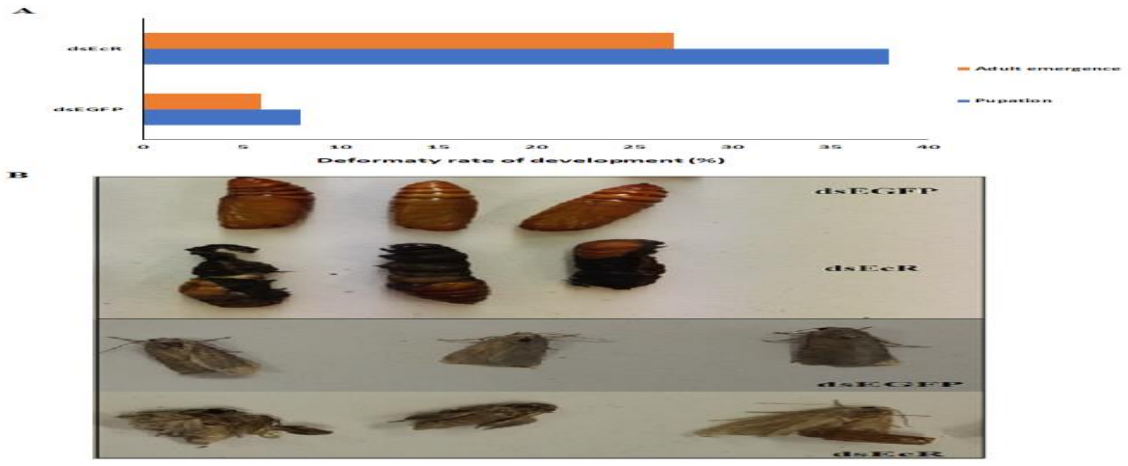

Figure 7. Deformity rate and abnormal adult emergence (A) Larval lethality rate in *H. armigera* after 10 days of feeding *dsEcR* compared to the *dsEGFP* control (B) The deformation of adult emergence in *dsEcR* treated insects compared to the *dsEGFP* control.

DISCUSSION

Sequence specific knockdown of mRNA transcripts by RNAi propose countless opportunities for crop protection, such as pest control and the reduction of crop damage by invading pathogens. An appropriate delivery strategy for dsRNAs can be a foremost restraint of RNAi studies for pests (Yu et al., 2013; Wynant et al., 2014; Scott et al., 2013). In this investigation, transformed bacteria encoding dsRNAs were used to identify the most efficient fragment. This is rapid, suitable and economical approach to produce dsRNA in bulk amount compared to the commercially available dsRNA synthesizing kits. Moreover, we demonstrated the induction of sequence specific RNAi effects in *H. armigera* by consumption of artificial diets coated with dsRNA. The dsRNAs fed through artificial diet triggered significant depletion of mRNA level in *H. armigera* compared to control group. A maximum 96% d*EcR*ease was detected on day 10 for *dsEcR-2* group, 93% d*EcR*ease for *dsEcR-1* after 10 days of feeding, and 89% d*EcR*ease in targeted transcripts was observed for *dsEcR-3* on day 10 through oral intake of each dsRNA. Our results were slightly different from previous study using oral consumption of dsRNA. Our q T-PCR data proposed that oral delivery to *H. armigera* could accomplish similar knockdown effects on as in other insects. For example, oral delivery of dsRNA to the termite *R. flavipes* reduced mRNA relative expression of *Cell-1* and *Hex-2* approximately 60% on day 2 (Zhou et al., 2008). Furthermore, a significant d*EcR*ease (42%) of *salivary gland nitrophorin 2* was demonstrated 48 hours after ingesting dsRNA in *R. prolixus* (Araujo et al., 2006). A study on *E. postvittana* and *S. frugiperda* also revealed that ingesting dsRNA could cause depletion of transcripts level in various pest orders (Tumer et al., 2006).

Based on the RNAi effect at the transcript level we would assume a high mortality rate in *dsEcR-1, dsEcR-2 and dsEcR-3* treated larvas as compared to control *egfp* group. Moreover, higher larval lethality was also observed in dsRNA feeding assay test targeting *HaHR3* gene of the *H. armigera* larvae (Xiong et al., 2013). In our findings, the mortality rate in *dsEcR*-2 treated larva is relatively high compared to the the study conducted on adult S. exigua targeting developmental gene (Tian et al., 2009). Additionally, the low mortality rate was observed in *ds-EcR-1* and *ds-EcR-3* test groups even after 10 days of feeding than a recent study conducted by *Malik et al., 2016* on white fly. There might be several reasons underwrite this phenomenon. First, larvae were used as the object of study as compared to adult flies used in the study conducted. Somewhat same results were demonstrated in research conducted by *Baum et al.*, 2007 for *WCR* feeding assay. To improve efficacy of RNAi, efficient approach for dsRNA delivery is required. Moreover, stabilization of dsRNA in the body of targeted insect (gut or hemocoel) is the key feature of Effective dsRNA delivery method. There are chances of dsRNA degradation by nucleases in gut lumen and some tissues of insects (Terenius et al., 2011). For instance, dsRNAse found in the saliva of *Lygus lineolaris* has been reported to catalyze specific cleavage of dsRNA (Allen et al., 2012). Similarly, dsRNA degradation was also recorded in Pea aphid *Acyrthosiphon pisum* after oral delivery of artificial diet coated with dsRNA (Christiens et al., 2014). A specific dsRNase in the midgut of *Bombyx mori* has been reported to play a central role in the degradation of RNA and DNA, primarily to protect against viral attack by digestion of viral nucleic acids (Liu et al., 2012). Four different types of dsRNase has been reported in *Schistocerca gregaria*, and RANi efficacy of dsRNAs can be improved by targeting dsRNAse 2 (Wynant et al., 2014). These reports proposed that body fluid of *Helicoverpa armigera* might have dsRNase activity. The dsRNase activities relied on the larval developmental stages and tissues. dsRNA targeting ultraspiracle gene of *Helicoverpa armigera* has revealed rapid digestion in midgut juice compare to hemolymph (Yang et al., 2014). Consequently, high dose of dsRNA or continuous oral delivery would be required to achieve effective knockdown. To determine the optimum dose of dsRNA in our study, different concentrations of bacterially expressed dsRNAs were mixed with artificial diet and fed to newly hatched caterpillar. Increased dose (15 µg) significantly increased RNAi efficacy and insecticidal activity. Such lethal activity of dsRNA is lower compared to the findings of other studies conducted on lepidopteran insects. For example, targeting β integrin subunit of *S. exegua* through bacteria expressing dsRNA caused only 50% mortality (Kim et al., 2015). RNAi against immunosuppressive chymotrypsin gene of *S. exegua*, resulted in higher larval lethality of 45% mortality even at higher concentration of dsRNA (Vatanparast and Kim, 2017). RNAi efficiency enhanced with increased dose of dsRNA in cotton bollworm *Pectinophora gossypiella* (Yang and Han, 2014). Decreased mortality rate of larvae revealed by this study might be due to the continuous oral delivery of dsRNAs for prolonged timespan compared to the above-mentioned study in which larvae were fed once.

Successful RNAi in *H. armigera* permitted us to employ it as a standard for developing a faster and easier approach to evaluate gene function. Since oral feeding of dsRNAs is a robust approach we could evaluate the effect of three *EcR* gene fragments in short time and found *EcR* to be a vital target for pest control. Since larvae were failed to gain weight and body size was significantly reduced feeding on *dsEcR*-2 coated artificial diet as compared to control (Figures 3 and 5), the siRNAs produced by this gene fragments among the three may play a key role in binding to the target mRNA in *H. armigera* resulting higher knockdown efficiency and larval letahlity. Larvae fed on *dsEcR*-1 and *dsEcR*-3 show reduced mortality rate as compared dsRNA coated diet. Data obtained from larval groups feeding on *dsEcR* showed similar kind of molting abnormality with larval-pupal intermediate in *H. aramigera*. (Figure 5A) showing lethal phenotype comparable to *B. mori* fed with *dsEcR* (Tian et al., 2010).

CONCLUSION

For a longtime, it was supposed that *EcR* is found in arthropods and specifically in insects (Nakagawa et al., 2010). But recent investigation has identified *EcR* homologous in nematodes having similar molting ability like insects (Graham et al., 2010). Meanwhile, nematodes also damage crops, targeting *EcR* might be a good option for nematode pest management. However, targeting *EcR* might have a biosafety problem in higher organisms. But, no *EcR* homologous has been found in higher animals (Nakagawa et al., 2010), so *EcR* gene can be carefully chosen for pest management. More notably, the present study established the usefulness of RNAi mechanism for precise and robust knockdown of a genetic elements in lepidopteran insects that are resistant to transgenic *Bt* plants. Discrete genomic and physiological changes are anticipated to occur between agriculturally harmful pest species, making fundamental the vigilant selection of genetic targets (s) for specific insect species, while also considering the adverse effects on non-target or beneficial insects that could encounter transgenic plants. Several considerations are thereby vital for the achievement of harmless and effective RNAi mediated insect control. Particular for screening insect associated genes for pest management, the transgenic bacteria expressing dsRNA is a very effective method because of its robustness and economical approach.

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